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(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : BROGLIE, Karen, Elizabeth [US/US]; BROGLIE, Richard, Martin [US/US]; 121 Bunting Drive, Wilmington, DE 19808 (US).

(74) Agents: MORRISSEY, Bruce, W. et al.; E.I. du Pont de Nemours and Company, 1007 Market Street, Wilmington, DE 19898 (US).

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(54) Title: OVEREXPRESSION OF CHITINASE IN TRANSGENIC PLANTS

(57) Abstract

The preparation of novel recombinant DNA constructs and their use in transforming plants to achieve overexpression of chitinase and thereby conferring resistance to various plant pathogenic fungi.

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TITLE

OVEREXPRESSION OF CHITINASE IN TRANSGENIC PLANTS

Field of the Invention

This invention relates to the preparation of novel recombinant DNA constructs used to introduce and overexpress chitinase polypeptide(s) in plants to confer resistance to plant pathogenic fungi, and to such transgenic plants and their seeds.

BACKGROUND OF THE INVENTION

The process by which plants protect themselves against potentially pathogenic microorganisms is dependent upon the timely accumulation of a number of host-synthesized proteins that are produced in response to pathogen attack. Associated with this process are the production of (i) certain lytic enzymes, such as chitinase and β-1,3-glucanase, which are capable of degrading fungal cell walls, (ii) phytoalexins (secondary metabolites that are toxic to bacteria and fungi), (iii) inhibitors of serine proteases and (iv) enzymes leading to the formation of physical barriers through modifications of the plant cell wall.

The hydrolytic enzymes chitinase and β-1,3-glucanase have been proposed to function in defense by causing extensive degradation of pathogen cell walls. Lysis of pathogenic fungal hyphae has been observed *in vivo* in plants infected with a number of vascular wilt pathogens. G. F. Pegg and J. C. Vessey (Physiol. Plant Path. 3:207-222, 1973) suggested that lysis of *Verticillium albo-atrum* hyphae in tomato was related to the host production of chitinase. *In vitro* studies demonstrated that extracts from fungal infected plants having chitinolytic activity were able to degrade isolated

fungal cell walls. D. H. Young and G. F. Pegg (Physiol. Plant Path. 19:391-417, 1981) showed that chitinase, isolated from infected tomato plants, released soluble fragments from the cell wall of Verticillium albo-atrum. Boller et al. (Planta 157: 22-31, 1985) demonstrated that purified bean chitinase attacked isolated cell walls of the bean pathogen Fusarium solani, f.sp. phaseoli. D. H. Young and G. F. Pegg also demonstrated that purified tomato glucanase was able to partially digest V. albo-atrum cell walls and that the digestion was increased synergistically in the presence of purified tomato chitinase. Ordentlich et al. (Phytopathology 78: 84-88, 1988) have shown that culture filtrates of the bacterium Serratia marcescens possess chitinolytic activity when grown on a medium containing cell walls of the plant pathogen Sclerotium rolsfii and its components, chitin and laminarin. Incubation of the culture filtrate with different substrates, including S. rolsfii cell walls, dry mycelium and washed mycelium, resulted in the release of N-acetyl-D-glucosamine residues indicating substrate degradation. Microscopic observations showed that crude chitinase preparations from the culture filtrate were able to degrade the growing hyphal tip.

Addition of chitinase to the soil, alone or in combination with other hydrolytic enzymes, has been shown to be inhibitory to other plant pathogens. P.M. Miller and D.C. Sands (Journal of Nematology, 9: 192-197, 1977) have studied the effects of a bacterial chitinase, obtained from a commercial source, on plant parasitic nematodes. Their results indicate that this enzyme is toxic to certain

nematodes, in particular, Tylenchorhynchus dubius. Their data indicate that this toxicity is greater in aqueous solution than in soil.

Endo-type chitinase activities have been observed in many species of higher plants including bean, pea, soybean, tomato, sunflower, melon, cotton, corn, wheat, barley and tobacco (Boller, T., Gehri, A., Mauch, F. and Vogeli, U. Planta (1983) 157:22-31; Shinshi, H., Mohnen, D. and Meins, F. (1987) Proc. Natl. Acad. Sci. USA 84:89-93; Leah, R., Mikkelsen, J.D., Mundy, J. and Svendsen, I. (1987) Carlberg Res. Commun. 52:31-37). In some instances, chitinase activity has been shown to increase in response to pathogen attack (Mauch, F., Hadwiger, L.A. and Boller, T. (1984) Plant Physiol. 76: 606-611; Pegg, G.F. and Young, D.H. (1981) Physiol.Plant Pathol. 19:371-382; Roby, D. and Esquerre-Tugaye, M.T. (1987) Plant Science 52:175-185). In general, the specific activity of the enzyme and its tissue specificity have been found to vary over a wide range when different plants are compared.

Plant chitinases have been purified from wheat germ, tomato, bean and pea. The enzymes isolated from these sources have been shown to correspond to basic proteins of approximately 30 kilodaltons (Boller, T. (1985) in "Cellular and Molecular Biology of Plant Stress" (Key, J.L. and Kosuge, T.,eds.) pp. 247-262, Alan R. Liss, Inc., N.Y. N.Y.). Chitinase cDNA and genomic clones have been isolated from bean, tomato, tobacco and potato. As discussed in Broglie et al. (1986) Proc. Natl. Acad. Sci. USA 83:6820-6824, an endochitinase from bean was found to

be encoded by a 1.2 kilobase messenger RNA comprised of a short, 33 base untranslated region followed by a 984 nucleotide open reading frame and 115 nucleotides of 3' untranslated RNA. The protein coding region specifies a 328 amino acid polypeptide which consists of a 27 amino acid residue signal peptide and the 301 residues of the mature chitinase polypeptide chain. The amino terminal signal sequence presumably functions in determining the vacuolar localization of the bean enzyme (Boller, T. and Vogeli, U. (1984) Plant Physiol. 74:442-444). The deduced amino acid sequence of the mature protein is consistent with the basic pI value reported for bean chitinase (Boller, T. (1985) in "Cellular and Molecular Biology of Plant Stress" (Key, J.L. and Kosuge, T. , eds.) pp. 247-262, Alan R. Liss, Inc., N.Y. N.Y.). Sequence comparisons indicate that the bean protein shares 70% homology with the tomato enzyme and 73% homology with the enzyme from tobacco.

The isolation of chitinase cDNA and genomic clones provides the opportunity to manipulate the expression of this protein and to evaluate the effect of this genetic modification on the fungal resistance of the derived plants. The involvement of chitinase in the defense of the plant against chitin-containing fungal pathogens is based upon the following indirect evidence and upon data generated from model, in vitro systems. First, while there is no known plant substrate for the enzyme, chitin is known to be a ubiquitous component of the cell walls of most fungi except the oomycetes (Wessels, J.G.H. and Sietsma, J.H. (1981) in "Plant Carbohydrates II" (Tanner, W. and Loewus, F.A., eds.) pp. 352-394, Springer-Verlag, N.Y., N.Y.). Second, the levels of enzyme have been

found to increase in plants infected with fungal pathogens (Mauch, F., Hadwiger, L. A. and Boller, T. (1988) Plant Physiol. 87:325-333). Thirdly, the purified enzymes from bean and tomato have been shown to degrade isolated fungal cell walls (Boller, T., Gehri, A., Mauch, F. and Vogeli, U. (1983) Planta 157:22-31; Young, D.H. and Pegg, G.F. (1982) Physiol. Plant Pathol. 21: 411-423). Fourth, purified bean chitinase has been found to inhibit the vegetative growth of the non-pathogenic test fungus, Trichoderma viride (Schlumbaum, A., Mauch, F., Vogeli, U. and Boller, T. (1986) Nature 324:365-367). In this particular assay system, fungus was grown on solid, agar-containing medium and purified bean chitinase or a protein extract from ethylene-treated bean was introduced into wells in the agar plate. Zones of growth inhibition were found to develop around wells containing purified chitinase or the bean protein extract. This effect was attributed to chitinase-catalyzed hydrolysis of newly formed chitin in the growing hyphal tips. More recently, an in vitro system to assess the susceptibility of various fungi to chitinase and β-1,3-glucanase (an additional lytic enzyme of plant origin which hydolyzes β-1,3-linked polymers of glucose) was developed using the purified enzymes from pea (Mauch, F., Mauch-Mani, B. and Boller, T. (1988) Plant Physiol. 88: 936-942). Protein extracts from fungal-infected pea pods inhibited the growth of 15 of the 18 fungi tested. These extracts were shown to contain high levels of chitinase and β-1,3-glucanase activity. Eight fungi were tested for growth inhibition by either of the enzymes alone or by a combination of the two enzymes. Of the fungi tested, only Trichoderma viride was susceptible to the action

of chitinase alone and only Fusarium solani f.sp. pisi was inhibited by β-1,3-glucanase alone. While the results of Schlumbaum et al. and Mauch et al. provide additional data supporting the role of chitinase in the defense response of the plant, a certain amount of caution must be exercised lest the data be overinterpreted. Extrapolation of data obtained from the in vitro assay to results anticipated in vivo is particularly tempting but should be avoided. Some limitations of the in vitro assay system are given below. It should be remembered that the test fungus employed by Schlumbaum et al., Trichoderma viride, is not a plant pathogen; indeed, Trichoderma species are known to be parasites of other fungi and as such have been utilized as effective biocontrol agents to inhibit the growth of plant pathogenic fungi (Chet, I. (1987) in "Innovative Approaches To Plant Disease Control" (Chet, I., ed.) pp. 137-160). While Mauch et al. found that Fusarium solani f.sp. pisi is sensitive to the presence of chitinase and glucanase during growth on agar plates, this fungus is nevertheless a successful pathogen of pea. Thus, the presence or absence of sensitivity to the two hydrolytic enzymes in the plate assay may have little bearing on the phytopathogenic properties of the fungus. Growth on nutritive agar media is distinguished from infection of plant tissue by the striking lack of specialized infection structures in the former case. The possibility surfaces that the composition of the fungal cell wall during vegetative mycelial growth differs from that of the fungal infection structures. In this regard, Mendgen et al. (Mendgen, K. Freytag, S., Lange, M and Bretschneider, K. (1986)

J. Cellular Biochem. (Suppl.) 10C:25) determined that in the rust fungi, different infection structures display different surface carbohydrate patterns. In the germ tube that recognizes the host cuticle, chitin is mainly found. In contrast, the structures of the rust fungi in the leaf (substomatal vesicles and infection hyphae) contain mainly β-1,3-glucans on their surface.

While the assay systems of Schlumbaum et al. and Mauch et al. provide further evidence for the involvement of chitinase in the defense response of the plant, it must be remembered that this lytic enzyme is only one part of the complex system evolved by the plant to combat pathogenic attack. Specific mechanisms are employed to physically restrict access of the invading fungus to the plant cell by strengthening existing barriers. Among these are lignification (Kohle, H., Young, D.H. and Kauss, H. (1984) Plant Sci. Lett. 33:221-230) and suberization (Espelie, K.E., Francheschi, V.R. and Kolattukudy P.E. (1986) Plant Phsiol. 81:487-492) of the plant cell wall and the accumulation of hydroxyproline-rich glycoprotein (Showalter, A.M., Bell, J.N., Cramer, C.L., Bailey, J.A., Varner, J.E. and Lamb, C.J. (1985) Proc. Natl. Acad. Sci. USA 82:6551-6555) as a structural component of the plant cell wall. In addition, tactics are used to weaken or destroy the invading fungus. Among these strategies are the synthesis of phytoalexins, secondary plant metabolites which are toxic to bacteria and fungi (Dixon, R.A., Day, P.M. and Lamb, C.J. (1983) in Advances in Enzymology and Related Areas of Molecular Biology (Meister, A. ed.) pp. 1-135 Wiley, New York), induction of the synthesis and accumulation of

proteinase inhibitors, potent inhibitors of serine proteases which are present in animals and microorganisms but lacking in plants (Ryan, C.A., Bishop, P.D., Walker-Simmons, M., Brown, W.E. and Graham, J.S. (1985) in Cellular and Molecular Biology of Plant Stress (Key, J.L. and Kosuge, T., eds.), pp. 319-334. Alan R. Liss, Inc., New York) and the synthesis of the lytic enzymes chitinase and β-1,3-glucanase which are capable of hydrolyzing fungal cell walls (Boller, T. (1985) in Cellular and Molecular Biology of Plant Stress (Key, J.L. and Kosuge, T., eds.), pp. 247-262. Alan R. Liss, Inc., New York). The relative importance of the individual components in determining the final outcome of the host-pathogen interaction (compatible or non-compatible) is not known.

Recent studies of the kinetics of the induction of plant defense transcripts indicate that the timing of the appearance of these proteins may play an important role in the resistance of the plant to pathogen attack. When French bean (Phaseolis vulgaris) is infected with an incompatible (β) and a compatible (Y) race of Colletotrichum lindemuthianum and the RNA from infected plants analyzed on Northern blots, a difference is observed in the appearance of transcripts for phytoalexin biosynthesis in the two interactions. In the incompatible interaction (host resistant), phenylalanine ammonia lyase and chalcone synthase mRNAs accumulate rapidly and early in infection, being localized mainly at the site of fungal infection. In contrast, in the compatible interaction (host susceptible), appearance of the RNAs is delayed and more widespread than in the

incompatible interaction (Bell, J.N., Ryder, T.B., Wingate, V.P.M., Bailey, J.A. and Lamb, C.J. (1986) Mol. Cell. Biol. 6:1615-1623). Even in a successful resistant reaction, the appearance of the different defense mechanisms is not synchronous. Studies of plant cell suspension cultures treated with fungal elicitors (fungal cell wall fragments) indicate that phytoalexins and protease inhibitors generally appear prior to the accumulation of the hydrolytic enzymes chitinase and glucanase and these proteins precede the synthesis of hydroxyproline-rich glycoprotein (Chappel, J., Hahlbrock, K. and Boller, T. (1984) Planta 161:475-480; Lawton, M. A. and Lamb, C. J. (1987) Mol. Cell Biol., 7:335-341; Hedrick, S. A., Bell, J. N., Boller, T. and Lamb, C. J. (1988) Plant Physiol., 86:182-186). Other modifications of the plant cell wall occur later in the sequence of the defense response.

In order to generate fungal resistant plants, applicants have modified the timing of expression of a bean endochitinase gene in transgenic plants. In healthy, uninfected plants, chitinase is normally present at low, basal levels. However, treatment with ethylene, fungal elicitors or infection with fungi results in an induction of enzyme activity (Boller, T. (1985) in Cellular and Molecular Biology of Plant Stress (Key, J. L., and Kosuge, T., eds.) pp. 247-262, Alan R. Liss, Inc., New York). The promoter region, containing the DNA sequence elements for inducible expression, has been removed from an endochitinase gene from Phaseolus vulgaris and replaced with a promoter fragment of the cauliflower mosaic virus (CaMV) 35S transcript in order to promote high level, constitutive expression and to eliminate the time necessary for induction of

chitinase activity in response to pathogen attack. The CaMV 35S promoter fragment controls the expression of a bean chitinase gene which encodes a polypeptide consisting of a 26 amino acid residue signal peptide and 301 amino acids of the mature chitinase polypeptide. Transgenic plants of the present invention containing this modified chitinase gene have been shown to display increased resistance to infection by the foliar pathogen, Botrytis cinerea and by the soil-borne pathogen, Rhizoctonia solani.

Suslow, T. V. and Jones, J. D. G. disclose the use of a bacterial chitinase gene for disease protection. A chitinase gene from Serratia marcescens has been transferred to various rhizobacteria (European Patent application 0157351 published October 9, 1985 and U.S. Patent 4,751,081 issued June 14, 1988). These bacteria, which are capable of colonizing the roots of host plants, are utilized to introduce chitinase into the soil rhizosphere. However, there are a number of disadvantages associated with this approach. For example, this approach is applicable mainly to soil borne pathogens and is dependent on the stability of the enzyme in the rhizosphere. Also, the inhibitory effects of a chitinase produced by rhizobacteria would not be selective against pathogenic fungi but would also be inhibitory to non-pathogenic fungi that inhabit the rhizosphere and are beneficial to the growth and development of the plant.

An article appearing in Journal of Cellular Biochemistry (March, 1986) discloses that scientists at Advanced Genetic Sciences, Oakland, California, have introduced the chitinase gene from S. marcescens into experimental plants in an attempt to make them resistant to fungi. Uncertainty as to the

subcellular localization and stability of the bacterial enzyme in plant cells makes the use of bacterial chitinase genes as a source of disease resistance unreliable. The coding region of Serratia chitinase includes a 23 residue amino terminal extension which serves as a signal for the secretion of the protein into the extracellular milieu of this gram negative bacterium. In transgenic tobacco plants, the signal peptide is at least partially cleaved to yield a protein form which co-migrates with purified Serratia marcescens chitinase (Taylor, J. L. et al., Mol. Gen. Genet. (1987) 210:572-577; Jones, J. D. G. et al., Mol. Gen. Genet. (1988) 212:536-542). Whether the correctly processed form of the bacterial enzyme is secreted into the plant intercellular spaces, analogous to the trafficking pattern in the microorganism has not been disclosed. Results obtained in translocation experiments with the E. coli lamB protein provide an example of the difficulties involved in predicting the subcellular localization of bacterial proteins introduced into eukaryotic cells. The lamB protein is an integral membrane protein of the E. coli outer membrane. When synthesized in an E. coli cell-free translation system supplemented with bacterial membrane vesicles, lamB is found to be integrated into the vesicle membrane. However, in the presence of canine microsomal membranes, lamB is translocated across the vesicle membrane. Thus, while the eukaryotic translocation machinery of the microsomal membrane is able to recognize the bacterial signal sequence, it is unable to recognize the stop-transfer signals required for membrane integration (Watanabe, M. et al., Nature (1986) 323:71-73). Whether the prokarotic signals for extracellular secretion of a

bacterial chitinase are correctly recognized in plant cells, or whether the bacterial polypeptide is trapped in the plant cell or alternatively targeted to some other intracellular compartment is not readily predicted.

In bean, chitinase is known to be synthesized as a precursor protein containing an amino terminal peptide extension (Broglie, K.E., Gaynor, J.J. and Broglie, R.M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:6820-6824). This signal sequence presumably functions in determining the vacuolar localization of the mature bean enzyme (Boller, T. and Vogeli, U. (1984) Plant Physiol. 74:442-444). The signal sequence of the bean chitinase polypeptide was obtained by comparing the amino acid sequence deduced from the nucleotide sequence of a chitinase cDNA clone with the N-terminal sequence of the purified protein. This analysis indicates that the bean chitinase encoded by clone pCH18 contains a 27 residue signal peptide (Broglie, K.E., Gaynor, J.J. and Broglie, R.M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:6820-6824). The primary sequence of this segment is consistent with the qualitative features shared by other "hydrophobic" presequences: a relatively hydrophilic amino terminus containing several basic residues, followed by an apolar middle segment containing at least 7 or 8 largely hydrophobic residues and a relatively hydrophilic COOH terminus ending in an amino acid bearing a small side chain (Verner, K. and Schatz, G. (1988) Science 241:1307-1313). The signal sequences of other plant vacuolar proteins have been reported (Graham, J.S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. and Ryan, C.A. (1985) J. Biol. Chem. 260:6555-6560; Cleveland, T.E., Thornburg, R.W. and Ryan, C.A. (1987) Plant Mol. Biol. 8:199-207).

In the construct of the present invention, the coding sequence for bean chitinase (specified by the chitinase gene of bean genomic clone λCH 5B) is preceeded by its cognate 26 amino acid residue signal peptide. In transgenic tobacco plants which harbor this construct, the bean chitinase precursor protein is found to be efficiently processed to the mature form of the enzyme. Immunoblots of soluble protein isolated from these plants show the presence of a protein band, immunoreactive with anti-chitinase IgG and identical in size with purified bean chitinase. Efficient recognition and cleavage of the bean signal peptide in the heterologous plant background is indicative of its translocation to the central vacuole of the plant cell. These results are contrasted with those observed for the chitinase from Serratia marcescens in tobacco plants, where multiple higher molecular weight bands observed in Western blots indicate inefficient recognition of the prokaryotic signal peptide or additional posttranslational modifications of the heterologous polypeptide chain (Jones, J. D. G. et al. (1988), Mol. Gen. Genet., 212:536-542).

International Patent Application WO 88/00976 published February 11, 1988 discloses the possibility of introducing a chitinase enzyme from bacteria into plants to disrupt the chitin integument of plant feeding insects. As discussed above, it is questionable whether a bacterial chitinase is effective in the transformation of plants to achieve resistance to pathogenic fungi. The chitin in the insect integument is highly cross-linked and therefore refractory to chitinase. In addition,

there is no disclosure of how to actually isolate, clone, and insert the chitinase gene to transform plants.

The present invention utilizes a genetically engineered chitinase gene consisting of a high level promoter, a signal sequence and a protein coding sequence, which functions in plants to provide protection against chitin-containing pathogens. While most plants contain natural chitinase genes, the final enzyme level and the rate of induction in response to pathogen attack can vary over a wide range depending upon the species and environmental conditions. In the present invention, transgenic plants have been produced which contain a chimeric chitinase gene in which the inducible, regulatory region (promoter) of a natural chitinase gene was replaced with a viral DNA fragment in order to promote high level expression and to eliminate the need for induction of chitinase activity in response to pathogen attack. The present invention also contains a DNA sequence which encodes a short signal peptide which is required to direct the mature chitinase enzyme to the central vacuole of the cell. Transgenic plants containing the DNA construct of the present invention have been shown to exhibit increased resistance to attack by fungal pathogens.

SUMMARY OF THE INVENTION

This invention discloses a novel DNA construct which when introduced into plants, confers resistance to plant pathogenic fungi. Such transgenic plants incorporate a high level promoter and a coding sequence for a plant chitinase polypeptide under

the control of the high level promoter.

Specifically, one aspect of the present invention is a recombinant DNA construct capable of transforming a plant comprising the following DNA fragments: (a) a high level promoter operably linked to (b) a coding sequence for a plant chitinase gene or effective sequence thereof, wherein said high level promoter causes the overexpression of the chitinase polypeptide transport thereby conferring resistance to plant pathogenic fungi. An advantage of this invention is that unlike genes from other sources, the plant genes may contain one or more signal sequences which facilitate transport of said chitinase polypeptide to a plant cell vacuole. Preferred high level promoters are derived from the genome of a plant virus, a plant, or from the T-DNA region of Agrobacterium tumafaciens. More preferred high level promoters include the 35S and 19S promoters of the cauliflower mosaic virus, the NOS and OCS promoters of the opine synthase genes of Agrobacterium, the promoter of the RUBP carboxylase small subunit gene, and the promoter from the chlorophyll A/B binding protein genes. Most preferred, by virtue of activity or ease of preparation, is the 35S promoter constituent of the cauliflower mosaic virus. Preferred coding sequences for a signal peptide include a plant signal peptide, a chitinase signal peptide, and a synthetic signal peptide whose DNA sequence encodes a peptide which allows efficient transport of a protein to a plant vacuole. More preferred, by virtue of activity or ease of preparation, is the DNA sequence coding for the bean chitinase signal peptide. Preferred coding sequences for chitinase polypeptides include those

derived from plants, while those more preferred would be the bean chitinase polypeptide. The most preferred recombinant DNA construct includes a high activity promoter from the 35S constituent of the cauliflower mosaic virus, a coding sequence for a plant signal sequence from a bean chitinase structural gene, and a coding sequence for a chitinase enzyme from a bean chitinase structural gene.

Another aspect of the invention involves a plant containing a recombinant DNA construct described above which renders the plant resistant to plant pathogenic fungi. Preferred monocotyledonous plants include corn, alfalfa, oat, millet, wheat, rice, barley, and sorghum, while preferred dicotyledonous plants include soybean, tobacco, petunia, cotton, sugarbeet, sunflower, carrot, celery, flax, canola, cabbage, cucumber, pepper, tomato, potato, oilseed rape, bean, strawberry, grape, and lettuce. Most preferred, by virtue of ease of preparation, are tobacco, tomato, canola and rice plants transformed with a recombinant DNA construct incorporating the high activity promoter of the 35S RNA transcript of the cauliflower mosaic virus, the plant signal sequence of the bean chitinase signal peptide, and the coding sequence of the bean chitinase structural gene.

Finally, seed obtained by growing a transgenic plant described above represents another embodiment of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a restriction map of bean genomic clone lambda CH5B and the 4.7 kb HindIII-EcoRI fragment containing a bean chitinase gene. The

arrows depict the sequencing strategy employed to obtain the nucleotide sequence of this fragment. The bold line shows the open reading frame encoding the chitinase polypeptide. The following symbols are used to represent restriction enzyme sites in the genomic clone: B, BamHI; E, EcoRI; H, HindIII.

Figure 2 is the nucleotide sequence of the 4.7 kb HindIII-EcoRl fragment containing the bean chitinase gene. The 981 bp open reading frame encodes the chitinase precursor polypeptide which consists or a 301 amino acid mature enzyme (amino acid residues 27-301) and a 26 amino acid signal peptide (amino acid residues 1-26). The open reading frame is preceded by approximately 2 kb of 5' flanking DNA and is followed by approximately 1.7 kb of 3' flanking DNA. The deduced amino acid sequence is shown below the corresponding triplet codons.

Figure 3 is a summary of the steps involved in the construction of pK35CHN. A DNA fragment comprising bases 2052-3550 of the chitinase gene was fused to a 965 bp fragment bearing 35S promoter sequences. Kanamycin resistance marker genes were then inserted to yield pK35CHN. The segment denoted by a single line designated pBR322 refers to plasmid DNA sequences donated by the vector, pBR322. The following symbols are used to represent restriction enzyme cleavage sites: B, BamHI; C, ClaI; E, EcoRI; H, HindIII; S, SalI.

Figure 4 describes the immunodetection of bean chitinase in protein extracts from transgenic tobacco plants. Antibodies raised against gel-purified bean chitinase were used to detect the presence of the bean protein in transgenic tobacco plants. Antigen-antibody complexes were visualized using

alkaline phosphatase conjugated goat anti-rabbit IgG and an alkaline phosphatase specific histochemical reaction. Lanes contain the following protein extracts: Lanes 1-8, protein extract from 8 individual transgenic tobacco plants containing the 35S-chitinase chimeric gene; Lane 9, protein extract from ethylene-treated bean seedlings; Lanes 10-11, protein extracts from transgenic tobacco plants lacking the chimeric chitinase gene.

Figures 5A and 5B show the effects of Rhizoctonia solani infection on root fresh weight of transgenic tobacco plants containing a chimeric bean chitinase gene (plants # 230, 238, 329, and 373). Plant #548 contains a kanamycin resistance gene and serves as a control in this study. Data points are the mean root fresh weight of 10 plants determined two weeks after inoculation. Figure 5A and Figure 5B represent two different experiments.

Figure 6 describes the survival of transgenic tobacco plants containing the chimeric chitinase gene (#373) in soil infected with the plant pathogen Rhizoctonia solani compared to control tobacco plants lacking the modified gene (#548) and grown under identical conditions. Seedlings were transplanted into soil infested with R. solani and allowed to grow for an additional 16 days. Disease progression was monitored by scoring seedling survival at intervals following infection.

Figure 7 describes the partial resistance of transgenic tobacco plants containing the modified chitinase gene to infection by the foliar pathogen Botrytis cinerea. Plants were inoculated with a suspension of conidia and the number and size of the lesions determined after development of disease

symptoms. Plant #548 lacks the chimeric gene and served as a control in this experiment; plants #230, #329 and #238 all contained the chimeric gene and showed a reduction in lesion size following infection.

Figure 8 is an outline of the binary transformation vector pMChAD. The chimeric chitinase gene is inserted into the vector as a Kpn I fragment. The vector contains the right (RB) and the left (LB) borders of the T-DNA of Agrobacterium tumefaciens. The border fragments delimit the segment of DNA which is stably incorporated into the host plant. The vector also contains a chimeric marker gene consisting of the nopaline synthase promoter fused to the bacterial Npt II gene conferring resistance to the antibiotic kanamycin, followed by the octopine synthase 3' region. This vector also contains a sulfonylurea herbicide resistant ALS gene.

Figure 9 describes the immunodetection of bean chitinase in protein extracts of transgenic tomato plants. Lanes contain the following protein extracts: Lanes 1 and 6, purified bean chitinase; Lanes 2-4, protein extracts from transgenic tomato plants containing the chimeric chitinase gene in the binary vector pMChAD; Lane 5, transgenic tomato plants lacking the chimeric chitinase gene.

Figure 10 describes the immunodetection of bean chitinase in protein extracts of transgenic oil seed rape. Lanes contain the following protein extracts: Lanes 1 and 8, purified bean chitinase; Lanes 2 and 7, transgenic tobacco plants containing the chimeric chitinase gene; Lane 9, wild type (WT) untransformed Brassica napus; Lanes 4-6, 3 individual transformed B. napus plants.

Figures 11A and 11B describe the increased survival rate and delay in symptom appearance observed when transgenic canola plants containing the chimeric chitinase gene are grown in soil infested with Rhizoctonia solani. The experiment was performed on pooled R1 seed of two independently isolated transgenic canola lines. Figure 11A and Figure 11B represent the results of two separate experiments.

Figure 12 describes the immunodetection of bean chitinase in protein extracts of transgenic rice cells. Lanes contain the following protein extracts: Lane 1, purified bean chitinase; Lanes 2-6, 5 individual kan^R rice callus samples transformed with pK35CHN; Lane 7, control untransformed rice callus. The arrow indicates the bean chitinase polypeptide in transgenic rice cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a genetically engineered nucleic acid fragment which, when introduced into plants confers resistance to plant pathogenic fungi. This novel DNA fragment consists of (a) a promoter region which specifies high level expression fused to the coding region of a plant chitinase gene, and (b) a coding sequence for a plant chitinase gene or effective sequence thereof, wherein said high level promoter causes the overexpression of the chitinase polypeptide transport thereby conferring resistance to plant pathogenic fungi. The chitinase enzyme catalyzes the hydrolysis of chitin (Boller, T., et al. (1983) 157:22), a β-1,4-linked N-acetyl glucosamine polymer and an important component of fungal cell walls (Bartnicki-Garcia, S. (1968) Ann. Rev. Microbiol. 22:87).

In the context of this disclosure, a number of terms shall be utilized. As used herein, the term "promoter region" refers to a sequence of DNA, usually upstream (5') of the coding sequence, which controls the expression of a coding region of a gene. A promoter region can include a recognition site(s) for the binding of RNA polymerase and/or other factors required for correct transcription initiation. The promoter region may also contain DNA sequences which are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological conditions. A "promoter fragment" constitutes a DNA sequence consisting of a promoter region. "Regulatory sequence", as used herein, refers to a nucleotide sequence located upstream (5'), within, and/or downstream (3') to a DNA sequence for a selected gene product whose transcription and expression is controlled by the regulatory sequence in conjuction with the protein synthesis apparatus of the cell. An "enhancer" is a DNA sequence which can operate in an orientation- and location-independent manner to stimulate the activity of a promoter. A transcriptional "stimulator" or "activator" is a DNA sequence which operates in an orientation-dependent manner to increase the activity of a promoter. "Tissue-specific promoters" as referred to herein are those that direct gene expression only in specific tissues such as roots, leaves and stems. The term "expression", as used herein, is intended to mean the translation to gene product from a gene coding for the sequence of the gene product. In the expression, a DNA chain coding for a gene product is first transcribed into a

complementary RNA which is called a messenger RNA and then, the thus transcribed RNA is translated into the above-mentioned gene product in conjunction with the protein synthesis apparatus of the cell. Expression which is constitutive producing multiple copies of mRNA and large quantities of the specified gene product continuously throughout the life cycle of the plant.

"Overexpression" refers to the production of a gene product in transgenic plants that exceeds levels of production in normal plants, including but not limited to constitutive or induced expression.

"Nucleic acid" refers to a large molecule which can be single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or a pyrimidine. In higher plants, deoxyribonucleic acid (DNA) is the genetic material while ribonucleic acid (RNA) is involved in the translation of information encoded by DNA into proteins. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA. As used herein the term "homologous to" refers to the complementarity between the nucleotide sequence of two nucleic acid molecules or between the amino acid sequences of two protein molecules. Estimates of such homology are provided by either DNA-DNA or DNA-RNA hybridization under conditions of stringency as is well understood by those skilled in the art [as described in Hames and Higgins (eds.) Nucleic Acid Hybridization, IKL Press, Oxford, U.K.]; or by the comparison of sequence similarity between two nucleic

acids or proteins. As used herein, "substantially homologous" refers to nucleic acid molecules which require less stringent conditions of hybridization than those for homologous sequences, and coding DNA sequence which may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter an amino acid, but not affect the functional properties of the protein encoded by the DNA sequence. "Effective sequence" of a DNA sequence coding for a protein, refers to a truncated version of the DNA sequence which encodes a peptide which is at least partially functional with respect to the utility of the original protein.

As used herein, "gene" refers to a segment of DNA that is involved in producing a polypeptide chain; including regulatory regions preceding and following the coding region as well as intervening sequences between individual coding segments. As used herein, "coding region" or "coding sequence" refers to a region of a gene or a DNA sequence that codes for a specific protein. As used herein, "plant chitinase gene" refers to a segment of plant DNA which codes for an enzyme with chitinolytic activity. The term "recombinant DNA construct" refers to a DNA fragment, linear or circular, in which a number of nucleotide sequences have been joined into a unique and novel construction, capable of being introduced into a plant cell, and containing a promoter fragment and DNA sequence coding for a selected gene product. As used herein, the term, "operably linked" refers to the chemical fusion of

two DNA fragments in a proper orientation and reading frame to be transcribed into functional RNA. The "translational start codon" refers to a unit of three nucleotides (codon) in a DNA sequence that specifies the initiation of the structural gene of protein sequence.

A "signal sequence" refers to a peptide extension of a polypeptide, which is translated in conjunction with the polypeptide, forming a precursor polypeptide, and is encoded by a product DNA sequence. In the process of synthesis and transport to a selected site within the cell, for example, the endoplasmic reticulum or the vacuole, the signal peptide is cleaved from the remainder of the polypeptide precursor to provide an active or mature protein. As used herein, "secretion" means the transfer of a polypeptide molecule into the intercellular space of a plant.

As used herein, "transformation" means processes by which cells/tissues/plants acquire properties encoded on a nucleic acid molecule that has been transferred to the cell/tissue/plant. "Transferring" refers to methods to transfer DNA into cells including, but not limited to, microinjection, microprojectile bombardment, permeabilizing the cell membrane with various physical (e.g., electroporation) or chemical (e.g., polyethylene glycol, PEG) treatments. As used herein, "protoplast" refers to a plant cell without a cell wall or extracellular matrix.

The techniques of DNA recombination used throughout this invention are known to those skilled in the art and are generally described in Maniatis

et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., 1982).

Isolation of a Bean Chitinase Gene

A genomic library from Phaseolus vulgaris var. SAXA was constructed in bacteriophage lambda EMBL 4 [Frischauf, A.M. et al J. Mol. Biol. (1983) 170:827]. Total DNA was isolated from etiolated bean leaves. Tissue was frozen in liquid nitrogen, ground to a fine powder and then transferred to a buffer consisting of 10 mM Tris-HCl, pH 7.6, 0.35 M NaCl, 1mM EDTA, 7 M urea, 2% sarkosyl and 5% phenol (2 ml per gram tissue). After stirring at room temperature for 10 minutes, the sample was centrifuged to remove insoluble material and the supernatant was extracted with a 3:1 mixture of phenol:chloroform until a clear interface was evident. The DNA sample was dialyzed against 2 changes of 4 liters 10 mM Tris-HCl, pH 8.0, 10 mM EDTA and 10 mM NaCl at 4°C for 4 hours. To the dialyzed material, 1 gram of CsCl was added per ml solution and ethidium bromide was added to 0.125 mg/ml. The DNA samples were centrifuged to equilibrium density in a VTi50 rotor at 50,000 rpm for 24 hours. The DNA band was isolated by side puncture of the tubes and was again subjected to CsCl/ethidium bromide density gradient centrifugation. The DNA was collected and concentrated by ethanol precipitation. The alcohol precipitate was centrifuged, washed with 80% ethanol and dissolved in 10 mM Tris-HCl, 1 mM EDTA, (TE buffer) pH 8.0.

Total bean DNA was subjected to partial digestion with the restriction enzyme Sau3A. 400 µg

DNA was incubated with 6.8 units Sau3A in 9 ml 6 mM Tris-HCl, pH 7.5, 50 mM NaCl, 6 mM $MgCl_2$ and 100 µg/ml bovine serum albumin (BSA) at 37°C for 1 hour. The digested DNA was purified by phenol/chloroform extraction and concentrated by ethanol precipitation. The DNA was collected by centrifugation, washed with 80% ethanol and dissolved in 500 µl TE buffer, pH 8.0. The sample was loaded onto a 38 ml 10-40% sucrose density gradient prepared in 20 mM Tris-HCl, pH 8.0, 1 M NaCl and 5 mM EDTA and centrifuged in an SW 27 rotor at 26,000 rpm for 24 hours at 15°C. Following centrifugation, 0.5 ml fractions were collected and 10 µl of each were analyzed on a 0.4% agarose gel. Fractions containing DNA migrating in the 10-20 kilobase (kb) size range were pooled, dialyzed extensively against TE buffer, pH 7.8 and concentrated by ethanol precipitation. The ethanol precipitate was centrifuged, washed with 80% ethanol and dissolved in TE buffer, pH 7.8 at 0.12 mg/ml.

λEMBL 4 vector DNA was prepared essentially as described in T. Maniatis, E. F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (1982). 100 µg DNA was digested to completion with 300 units BamHI in 25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 100 µg/ml BSA for 2 hours at 37°C. The digested sample was purified by phenol/chloroform extraction and ethanol precipitation. The DNA was centrifuged, washed with 80% ethanol and dissolved in TE buffer, pH 8.0 at a concentration of 150 µg/ml. $MgCl_2$ was added to 0.01 M and the sample incubated at 42°C for 1 hour. The annealed lambda EMBL 4 DNA was loaded onto a 38

ml 10-40% sucrose gradient prepared in 20 mM Tris-HCl, pH 8.0, 1 M NaCl and 5 mM EDTA. The sample was centrifuged in an SW 27 rotor at 26,000 rpm for 24 hours at 15°C. Following centrifugation, 0.5 ml fractions were collected. A 15 µl aliquot of every third fraction was heated at 68°C to disrupt the cohesive arms and then subjected to electrophoresis on a 0.5% agarose gel. Fractions containing the left and right arms but lacking uncut DNA or stuffer fragment were pooled, dialyzed extensively against TE buffer, pH 8.0 and concentrated by ethanol precipitation. The precipitated DNA was centrifuged, washed with 80% ethanol and dissolved in TE buffer, pH 8.0 at 0.24 mg/ml.

λEMBL 4 arms and the 10-20 kb fragments of bean DNA were ligated at a molar ratio of 1.3:1 (arms:inserts) in 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT and 100 µg/ml BSA containing 0.5 µg/ml DNA and 133 units T4 DNA ligase/ml. After a 16 hour incubation at 15°C, the ligation mixture was packaged into viable phage particles using the Packagene system available through the Promega Corporation (2800 S. Fish Hatchery Road, Madison, WI 53711). The entire packaging mixture was plated out using E. coli strain LE392supF as host. The unamplified library consisting of 5×10^5 recombinant plaques was screened for chitinase genomic sequences using a nick-translated EcoRI insert of a bean chitinase cDNA clone (pCH18), isolated as described in Broglie, K. E., Gaynor, J. J. and Broglie, R. M. (1986) Proc. Natl. Acad. Sci. USA 83:6820-6824. Six positive clones were obtained and subjected to plaque purification. The DNA of these purified clones was

digested with the restriction enzymes EcoRI, BamHI, HindIII and KpnI. The derived restriction maps indicate that the genomic clones comprise three different bean chitinase genes. The DNA fragments harboring the chitinase genes were identified by hybridization of Southern blots of restricted phage DNA to nick-translated pCH18 insert DNA.

A 4.68 kb HindIII-EcoRI fragment of bean genomic clone λCH5B was subcloned into a plasmid vector to allow determination of the nucleotide sequence of this chitinase gene. 3 µg of plasmid pCH31, containing an 8.4 kb EcoRI fragment of λCH5B, was digested with 11 units HindIII and 7.5 units EcoRI in 30 µl 25 mM Tris-HCl, pH 7.8, 75 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 100 µg/ml BSA at 37°C for 2 hours. After this time, 1/50 volume of 0.1M DTT, 1/10 volume of 0.5 mM dGTP, dATP, dTTP, dCTP and 5 units Klenow fragment of E. coli DNA polymerase I were added and the sample incubated at room temperature for 30 minutes. A 5 µl aliquot was loaded on a 0.7% low melting point agarose gel in 40 mM Tris-acetate, 1 mM EDTA, pH 8.2 (TAE buffer). When the bromophenol blue dye marker had migrated three-quarters of the way into the gel, electrophoresis was stopped and the gel stained in 1 µg/ml ethidium bromide and destained in H_2O. The 4.7 kb band was excised from the gel, melted at 68°C and ligated to 0.14 µg SmaI digested pEMBL 8+ DNA in 200 µl 25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM DTT, 0.25 mM spermidine, 1 mM ATP, 1.25 mM hexamine cobalt chloride and 10 µg/ml BSA containing 4 units T4 DNA ligase. After a 16 hour incubation at 12.5°C, 10 µl of the ligation mixture was used to transform E. coli strain JM 101. Transformants were selected on

Luria-Bertani (LB) (Table IV) media containing 100 µg/ml ampicillin. Plasmid DNA was isolated from 1.5 ml cultures of individual transformants, essentially as described in Maniatis et al, pg 368. The mini prep DNA was digested with the restriction enzymes EcoRI and BglII to determine the orientation of the inserted fragment in pEMBL 8+.

Plasmid DNA was isolated from transformants containing the 4.68 kb genomic fragment in both orientations in the vector, pEMBL 8+ (designated pCH34 and pCH35). pCH34 and pCH35 DNA was then purified by two cycles of CsCl/ethidium bromide density gradient centrifugation. A nest of ordered deletions was created across the insert sequence using a modification of the procedure of Barnes, W. M., Bevan, M. and Son, P. H. (1983) Methods in Enzymol. 101:98. 20 µg of each supercoiled DNA was incubated with 0.2 µg DNAse I in 100 µl 4 mM Tris-HCl, pH 7.9, 0.125 M NaCl, 20 mM $MgCl_2$, 0.5 mg/ml ethidium bromide and 60 µg/ml BSA for two hours at room temperature. Following the nicking reaction, the DNA was purified by phenol/chloroform and ether extraction and precipitation with ethanol. The alcohol precipitates were collected by centrifugation, washed with 80% ethanol and dissolved in 100 µl 66 mM Tris-HCl, pH 8.0, 77 mM NaCl, 5 mM $MgCl_2$ and 10 mM DTT. In order to widen the nick to a gap, 50 units of Exonuclease III were added to each and the samples incubated at room temperature for 7 minutes. The digestions were quenched by heating the samples to 70°C for 10 minutes. After cooling to room temperature, 60 µl H_2O, 5 µl 100 mM Tris-HCl, pH 8.0, 3 M NaCl, 60 mM $MgCl_2$, 60 mM $CaCl_2$, 5 mM EDTA and 1 unit nuclease Bal 31 were added and the

incubations allowed to proceed at room temperature for 5 minutes. The linearized DNA was extracted with phenol/chloroform and precipitated with ethanol. Deletions were created by digesting 10 µg of each DNA sample with 50 units BamHI in 100 µl 25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 100 µg/ml BSA at 37°C for 45 minutes. The ends were repaired by the addition of 2 µl 0.1 M DTT, 10 µl 0.5 mM dGTP, dATP, dTTP, dCTP and 10 units Klenow followed by a 30 minute incubation at room temperature. The samples were extracted with phenol/chloroform, precipitated with ethanol and dissolved in 100 µl TE buffer, pH 8.0. The deleted plasmids were recircularized by incubation of 10 µl of each sample with 2 units T4 DNA ligase in 50 µl ligation buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT and 100 µg/ml BSA) for 16 hours at 12°C. A 10 µl aliquot of each sample was used to transform E. coli strain JM 101. Transformants were selected on LB plates containing 100 µg/ml ampicillin.

Single stranded DNA was isolated from individual transformants upon superinfection with the F1 phage IR1. 1.5 ml aliquots of LB broth containing 100 µg/ml ampicillin and 1.7×10^8 phage/ml were inoculated with single colonies and the bacteria allowed to grow at 37°C overnight. Single stranded DNA was obtained from the liquid cultures using the procedure of Dente et al (Nucleic Acids Res. (1983) 11:1645-1655). The extracted DNA was analyzed on 0.75% agarose gels and samples containing DNA progressively shortened by approximately 250 bases were sequenced using the dideoxy chain termination procedure (Sanger, F., Nicklen, S. and Coulson, A. R.

[1977] Proc. Natl. Acad. Sci. USA 74:5463-5467). The products of the sequencing reactions were resolved on buffer gradient sequencing gels (Biggin, M. D., Gibson, T. J. and Hong, G. F. [1983] Proc. Natl. Acad. Sci. USA 80:3963-3965).

Figure 1 shows a restriction map of the approximately 17 kb of bean DNA cloned in λCH5B. Figure 1 also shows a restriction map of the 4.7 kb HindIII-EcoRI fragment which contains a bean chitinase gene and hybridizes to the chitinase cDNA clone, pCH18. The arrows in the figure represent the sequencing strategy used to obtain the complete nucleotide sequence of this DNA fragment.

The DNA sequence of this fragment and the deduced amino acid sequence of the chitinase precursor polypeptide is shown in Figure 2. The polypeptide is encoded by a single uninterrupted open reading frame consisting of 981 base pairs. This region is surrounded by 2.03 kb of 5' flanking DNA and 1.67 kb of 3' flanking DNA.

Construction of pK35CHN

A deletion subclone of pCH35, produced as described in the preceding section for DNA sequence analysis, was utilized as a starting point in the construction of pK35CHN. pCH35Δ6 contains 600 base pairs (bp) of 5' flanking DNA, a 981 bp open reading frame consisting of the mature chitinase polypeptide and a 26 amino acid residue signal peptide and 1670 bp of 3' flanking DNA. To generate a fragment containing only the protein coding region and 3' flanking sequences, pCH35Δ6 plasmid DNA was first linearized by digestion with the restriction enzyme HindIII (2 units/μg DNA) in medium salt buffer (25 mM

Tris-HCl, pH 7.8, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 100 µg/ml BSA) at 37°C. The digested DNA was purified by phenol/chloroform extraction and ethanol precipitation.

Linearized pCH35Δ6 DNA was then incubated at 0.1 mg/ml in 25 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 12 mM $MgCl_2$, 12 mM $CaCl_2$, 1 mM EDTA and 250 µg/ml BSA containing 0.05 units nuclease Bal 31/µg DNA. Incubation was allowed to proceed at 30°C. Aliquots were removed at timed intervals and quenched by the addition of EGTA to 20 mM. 5 µl of each time point was diluted with 8.5 µl of H_2O and 1.5 µl of low salt buffer (250 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 10 mM DTT and 1 mg/ml BSA) and 5 units of HindII were added. After two hours at 37°C, the samples were subjected to electrophoresis on a 1% agarose gel. The course of the Bal 31 digestion was monitored through the change in the mobility of the 1.2 kb band generated from the 5' end of the insert by HindII digestion. The sample which showed a loss of approximately 600 bp from the 1.2 kb band was purified by phenol/chloroform extraction and ethanol precipitation. The DNA was repaired in an end filling reaction consisting of 0.1 mg/ml DNA in 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT, 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 um dTTP, 50 µg/ml BSA and 0.8 units Klenow/µg DNA. After 30 minutes at room temperature, the reaction was terminated by heating to 70°C for 5 minutes. 2 µg of the blunt-ended DNA was ligated to 0.75 µg phosphorylated HindIII linkers in 42 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 2 mM ATP, 1 mM spermidine, 3 mM DTT and 70 µg/ml BSA containing 2 units T4 DNA ligase. After 16 hours at 15°C, the ligation mixture was extracted

with phenol/chloroform and precipitated with ethanol. The DNA was collected by centrifugation, washed with 80% ethanol, and dissolved in medium salt buffer. The DNA was digested in a total volume of 50 µl buffer with 80 units HindIII at 37°C. After 4 hours, the salt concentration was increased to 100 mM NaCl, 20 units of the restriction enzyme BglII were added, and incubation resumed at 37°C for 2 additional hours. The sample was concentrated by ethanol precipitation and the precipitate dissolved in 12 µl TE buffer, pH 8.0. 3 µl of gel loading buffer (25% Ficoll, 0.25% bromophenol blue and 0.25% xylene cyanol) was added and the sample run on a 0.8% low melting point agarose gel in TAE buffer. After electrophoresis, the gel was stained in 1 µg/ml ethidium bromide, destained in H_2O and visualized under long wave UV light. The HindIII-BglII fragment was excised from the gel, the agarose melted at 68°C, and the DNA ligated to 0.48 µg of HindIII-BamHI digested pEMBL 8+ in 170 µl ligation buffer containing 3 units T4 DNA ligase. After 16 hours at 12.5°C, 10 µl of the ligation mixture was used to transform E. coli strain JM 101. Transformants were selected on LB plates containing 100 µg/ml ampicillin.

Transformants were analyzed by nucleotide sequence analysis in order to define the 5' end point of the DNA fragment containing the chitinase coding and 3' untranslated region. Single colonies were inoculated into 1.5 ml LB broth containing 100 µg/ml ampicillin and 1.7×10^8 IR1 phage/ml. After 16 hours at 37°C, single stranded DNA was isolated from the liquid cultures using the procedure of Dente et al. (Nucleic Acids Res. (1983) 11: 1645-1655).

Single stranded DNA from selected transformants was sequenced by the dideoxy chain termination procedure of Sanger et al. (Proc. Natl. Acad. Sci. USA (1977) 74:5463-5467) and the products of the sequencing reaction resolved on buffer gradient sequencing gels according to the procedure of Biggin et al. (Proc. Natl. Acad, Sci. USA (1983) 80:3963-3965). From this analysis, two clones were selected, 641 and 695. In 641, the 5' end point of the chitinase fragment is located at +5 relative to the transcriptional start site. 641 thus contains 21 bp of 5' untranslated sequence, 981 bp of the complete open reading frame encoding chitinase and 515 bp of 3' flanking DNA. 695 is identical to 641 except that the 5' endpoint of the chitinase fragment is found at +23. Since it was not initially known whether the amount of 5' untranslated DNA would influence expression of bean chitinase through an effect on the stability of the mRNA, both 641 (which contains 21 bp of 5' untranslated DNA) and 695 (which contains 3 bp of 5' untranslated DNA) were used to construct a chimeric chitinase gene. Since the protein coding region is identical in both cases, the final polypeptide product derived from the 641 and 695 fragments will be identical also. Moreover, as detailed later, no systematic difference could be found between the constructs derived from 641 or 695 in terms of the amount of bean chitinase polypeptide produced in transgenic plants. Since the level of bean chitinase produced in transgenic plants is not appreciably influenced by the amount of chitinase 5'untranslated sequence present in the chimeric gene, other chitinase coding, 3' end fragments with variable amounts of 5' untranslated sequence may also be used.

Plasmid DNA was isolated from 10 ml liquid cultures of clones 641 and 695 using a scaled down version of the alkaline-SDS lysis procedure of Birnboim, H.C. and Doly, J. (Nucleic Acids Res. (1977) 7:1513-1523). 1 µg of each DNA was digested with 6 units XhoII in 30 µl 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.01% Triton X-100 and 100 µg/ml BSA at 37°C for 2 hours. After this time, 5 ml of 10 x medium salt buffer and 30 units of HindIII were added, the sample volumes adjusted to 50 µl with H_2O and incubation continued at 37°C for 2 hours more. One-fifth volume of gel loading buffer was added to each sample and 15 µl of the clone 641 and 695 digests were loaded onto a 0.75% low melting agarose gel. Following electrophoresis, the gel was stained in 1 µg/ml ethidium bromide for 20 minutes, destained for 10 minutes in H_2O and the 1.5 kb HindIII-XhoII fragments excised.

The starting vector used to generate the 35S-chitinase constructs, pK35CHN641 and pK35CHN695, is termed pK35CAT. pK35CAT has been deposited with the American Type Culture Collection under the terms of the Budapest Treaty and has the deposit identification number ATCC68174. This plasmid in turn was constructed from the original vector, pKNK (ATCC 67284). pKNK contains in pBR322, a neomycin phosphotransferase II (NPT II) promoter fragment, a nopaline synthase (NOS) promoter fragment, the coding region of neomycin phosphotransferase II and the polyadenylation signals of the nopaline synthase gene. The 320 bp ClaI-BglII NPT II promoter fragment was obtained from the NPT II gene of the transposon Tn5 (Beck, E., Ludwig, G., Auerswald, E.A., Reiss, B. and Schaller, H. (1982) Gene 19:327-336). This segment was derived from a

HindII-BglII fragment by conversion of the HindIII site to a ClaI site through linker addition. The NPT II promoter fragment is followed by a 296 bp nopaline synthase promoter fragment (corresponding to nucleotides -263 to +33) (Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H. J. (1982) J. Appl. Genet. 1:561-574). This was obtained by the creation of a PstI site at the ATG initiation codon and subcloning of the Sau3A-PstI fragment behind the NPT II segment. The NOS promoter is followed by a 998 bp HindIII-BamHI sequence containing the NPT II coding region. The NPT II coding region was obtained from the transposon Tn5 (Beck, E., Ludwig, G., Auerswald, E.A., Reiss, B. and Schaller, H. (1982) Gene 19:327-336) by the creation of HindIII and BamHI sites at nucleotides 1540 and 2518, respectively. The NPT II structural region is then followed by a 702 bp BamHI-ClaI fragment corresponding to the 3' end of the nopaline synthase gene (nucleotides 848 to 1550) (Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H.J. (1982) J. Mol. Appl. Genet. 1:561-574). The remainder of pKNK consists of pBR322 sequences from 29 to 4361.

A physical map of pK35CAT is shown in Figure 3 and its construction outlined in Lin, W., Odell, J.T. and Schreiner, R.M. (1987) Plant Physiol. 84: 856-861. pK35CAT is a pBR322 based construct which contains a chimeric gene consisting of the 35S promoter of cauliflower mosaic virus, the protein coding region of chloramphenicol acetyl transferase (CAT) and the polyadenylation signals of the nopaline synthase gene. The 35S promoter fragment of pK35CAT was obtained from a 1.15 kb BglII segment of the CaMV genome (corresponding to sequences -941 to +208 relative to the 35S transcription start site) cloned in the plasmid vector pUC13 (Odell, J.T.,

Nagy, F. and Chua, N-H., (1985) Nature 313: 810-813). This plasmid was linearized with the restriction enzyme SalI and the 3' end of the fragment shortened by digestion with nuclease Bal31. Following the addition of HindIII linkers, the plasmid DNA was recircularized. From nucleotide sequence analysis of the isolated clones, a 3' deletion fragment was selected with the HindIII linker positioned at +21 relative to the transcription start site. The 35S promoter fragment was isolated as an EcoRI-HindIII fragment and substituted for the EcoRI-HindIII frament of pKNK containing NPT II and NOS promoter sequences to give the plasmid pK35K.

The chloramphenicol acetyl transferase coding region of pK35CAT was obtained as a 975 bp Sau3A fragment from pBR325. The 5' protruding ends were filled in by reaction with the Klenow fragment of DNA polymerase I and the blunt-ended fragment ligated into a similarly blunt-ended SalI site of pGEM2. A selected clone, pGCAT9, contains the insert oriented such that the HindIII and BamHI sites of the polylinker are located 5' and 3' respectively to the CAT coding region. The CAT coding region was isolated from this clone by HindIII-BamHI digestion and substituted for the NPTII coding region of pK35K. The resultant construct, termed pK35CAT, also contains the NOS 3' end fragment which remains unaltered in the conversion of pKNK to pK35CAT.

The chitinase coding and 3' end fragment, obtained by HindIII-XhoII digestion of clones 641 and 695 indicated above, were next cloned in the parent vector pK35CAT in place of the CAT coding region. The 975 bp CAT coding sequence was excised by combined HindIII and BamHI digestion of the plasmid

in medium salt buffer. Two 0.5 µg aliquots of the digested DNA were electrophoresed on a 0.75% low melting agarose gel in TAE buffer. The vector bands containing the 35S promoter and NOS 3' end fragment in pBR322 were excised, combined with the 1.5 kb fragments of clones 641 and 695 and ligated in 150 µl ligation buffer containing 3 units T4 DNA ligase for 16 hours at 12.5°C. 10 µl of each sample was used to transform E. coli strain HB 101. Transformants were selected on LB media containing 100 µg/ml ampicillin. Plasmid DNA was isolated from selected transformants and characterized by restriction enzyme analysis. Those transformants which were found to contain the expected fragments upon SphI digestion were designated p35CHN641 or p35CHN695, depending upon the source of the chitinase coding, 3' end fragment.

p35CHN641 and p35CHN695 plasmid DNA was isolated by the alkaline-SDS lysis procedure and purified by CsCl/ethidium bromide density gradient centrifugation. 10 µg of each plasmid was digested with a two fold excess of EcoRI in 25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 100 µg/ml BSA containing 5 units calf intestinal alkaline phosphatase for 1.5 hours at 37°C. After this time, 1/10 volume 1 M Tris-HCl, pH 8.8 and 5 units more alkaline phosphatase were added and the samples incubated at 55°C for 30 minutes. The treatments were quenched by the addition of EDTA to 10 mM, followed by heating to 70°C for 5 minutes. The digested DNAs were purified by phenol/chloroform extraction, precipitated with ethanol and dissolved in sterile H_2O at 0.2 µg/µl. A 0.4 µg aliquot of each vector was combined with 0.1 µg of a DNA fragment containing two kanamycin resistance genes in

25 µl ligation buffer containing 1 unit T4 DNA ligase. The 3.5 kb drug resistance marker consists of a bacterial NPTI and a chimeric NOS:NPTII:OCS gene. The NPTI gene confers kanamycin resistance in E. coli and A. tumefaciens while the NOS:NPTII:OCS gene confers kanamycin resistance to plant cells. After 16 hours at 12.5°C, a 5 µl aliquot of each ligation was used to transform E. coli strain HB 101 cells. Transformants were selected on LB agar containing 100 µg/ml kanamycin. The presence of the EcoRI fragment in individual transformants was confirmed by restriction enzyme digestion of isolated plasmid mini-prep DNA. Digestion with HindIII additionally permitted determination of the orientation of the inserted kanamycin resistance marker fragment.

E. coli strains HB101 carrying the plasmids pK35CHN641 and pK35CHN695 were deposited September 23, 1988 in American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A under the terms of the Budapest Treaty. The deposit identification numbers are ATCC67811 and 67812, respectively.

Although the construct of the present invention contains the coding region and 3' end of a bean chitinase gene fused to a DNA fragment bearing cauliflower mosaic virus 35S promoter DNA sequences, it is also possible to modify the expression of bean chitinase through the use of other regulatory DNA sequence elements (synthetic and natural) positioned 5' and 3' to the chitinase coding sequence. Other constitutive promoters which function in plants (e.g. nopaline synthase promoter (Depicker, A.,

Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H. M. (1982) J. Mol. Appl. Genet. 1:561-573; Sanders, P.R., Winter, J.A., Barnason, A.R., Rogers, S.G. and Fraley, R.T. (1987) Nucleic Acids Res. 15:1543-1557; Harpster, M.H., Townsend, J.A., Jones, J.D.G., Bedbrook, J. and Dunsmuir, P. (1988) Mol. Gen. Genet. 212:182-190), the 19S promoter of CaMV (Lawton, M.A., Tierney, M.A., Nakamura, I., Anderson, E., Komeda, Y., Dube, P., Hoffman, N., Fraley, R.T. and Beachy, R.N. (1987) Plant Mol. Biol. 9:315-324), the 1' and 2' divergent promoters of A. tumefaciens TR-DNA (Velten, J., Velten, L., Hain, R. and Schell, J. (1984) EMBO J. 12:2723-2730; Velten, J. and Schell, J. (1985) Nucleic Acids Res. 13:6981-6998; Harpster, M.H., Townsend, J.A., Jones, J.D.G., Bedbrook, J. and Dunsmuir, P. (1988) Mol. Gen. Genet. 212:182-190), etc.) may be used if these prove to be of sufficient strength. Alternatively, a constitutive promoter may be used in combination with a transcriptional stimulator or enhancer sequence (e.g. the octopine synthase enhancer (Ellis, J.G., Llewellyn, D.J., Dennis, E.S. and Peacock, W.J. (1987) EMBO J. 6:11-16; Ellis, J.G., LLewellyn, D.J., Walker, J.C., Dennis, E.S. and Peacock, W.J. (1987) EMBO J. 6:3203-3208), the first intron of the maize Adh1 gene (may provide reference), the 35S transcription stimulator (Kay, R., Chan, A., Daly, M. and McPherson, J. (1987) Science 236:1299-1302; Ow, D.W., Jacobs, J.D. and Howell, S.H. (1987) Proc. Natl. Acad. Sci. USA 84:4870-4874), etc.) in order to achieve the desired level of expression. Stronger expression of the chitinase polypeptide, driven by the 35S promoter of CaMV, may be achieved by the

duplication of 35S promoter sequences (Kay, R., Chan, A., Daly, M. and McPherson, J. (1987) Science 236:1299-1302).

Tissue or developmentally specific promoters may also be employed. The use of promoters such as those derived from ribulose bisphosphate carboxylase small subunit (rbcS) genes (Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G. and Chua, N-H. (1985) Nature 315:200-204; Dean, C., van den Elzen, P., Tamaki, S., Black, M., Dunsmuir, P. and Bedbrook, J. (1987) Mol. Gen. Genet. 206:465-474), of the chlorophyll a/b binding (Cab) protein genes (Jones, J.D.G., Dunsmuir, P. and Bedbrook, J. (1985) EMBO J. 10:2411-2418; Simpson, J., Timko, M.P., Cashmore, A.R., Schell, J., VanMontagu, M. and Herrera-Estrella, L. (1985) EMBO J. 4: 2723-2729) would optimize production of chitinase in leaf tissue to specifically combat foliar pathogens. Similarly, promoter sequences derived from root or stem-specific (Goldberg, R.B. Science 240:1460-1467) genes would provide preferential expression in these tissues and may thus provide protection against root and stem rot pathogens. Promoters obtained from developmentally regulated genes (Goldberg, R.B. (1988) Science 240:1460-1467); St. Schell, J. (1987) Science 237:1176-1183; Rosahl,S., Schell, J. and Willmitzer, L. (1987) EMBO J. 6:1155-1159; Sanchez-Serrano, J., Schmidt, R., Schell, J. and Willmitzer, L. (1986) Mol. Gen. Genet. 203:15-20; Chen, Z.-L., Pan, N.-S. and Beachy, R.N. (1988) EMBO J. 7: 297-302) may allow timing of the expression of the chimeric chitinase gene to coincide with developmental stages of the plant which are particularly susceptible to attack by fungal pathogens. As discussed above, if the desired

tissue or developmentally specific promoter proves to be of insufficient strength, one may combine this element with a transcriptional activator or stimulator.

Increasing evidence indicates that 5' untranslated leader segments can influence gene expression by regulation of mRNA translation. Kozak (1988, Mol. Cell. Biol. 8:2737-2744) has discussed the importance of the lack of secondary structure in and the length of the 5' leader. Lutke et al. (1987) have proposed an optimal context for ATG initiation codons in plant mRNAs (Lutke, H.A., Chow, K.C., Michel, F.S., Moss, K.A., Kern, H.F. and Scheele, G. (1987) EMBO J. 6:43-48). The nucleotide sequence of the 5' untranslated segment may also influence translational efficiency. The 5' untranslated region of several plant virus RNAs have been found to increase the expression of the reporter RNA to which they are linked (Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C. and Wilson, T.M.A. (1987) Nucleic Acids Res. 15:3257-3273; Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C. and Wilson, T.M.A. (1987) Nucleic Acids Res. 15:8693-8711; Jobling, S.A. and Gehrke, L. (1987) Nature 325:622-625). In this regard, a 5' leader sequence which stimulates translation of the chimeric chitinase gene may be inserted between the promoter region and the DNA segment which encodes the chitinase polypeptide.

Other termination signals may be used in place of the cognate chitinase 3' end. Alternate 3' untranslated sequences may contribute to increased stability of the mRNA thus facilitating strong expression of the chitinase polypeptide in transformed plants. The role of 3' sequences in

influencing the turnover of mRNAs has been documented in other eukaryotic systems (Simcox, A.A., Cheney, C.M., Hoffman, E.P. and Shearn, A. (1985) Mol. Cell. Biol. 5:3397-3402; Shaw, G. and Kamen, R. (1986) Cell 46:659-667; Petersen, R. and Lindquist, S. (1988) Gene 72:161-168; Brewer, G. and Ross, J. (1988) Mol. Cell. Biol. 8:1697-1708).

The DNA sequence elements mentioned above may be used alone or in different combinations to optimize levels of the chitinase polypeptide and to achieve the desired pattern of chitinase expression in plant cells. The aim of the final construct, when present in transformed plants, would be to provide either maximal broad-range resistance to all chitin-containing fungal pathogens or alternatively, resistance to a more selective group of fungi which contain chitin in their cell wall. In the latter approach, the target pathogens may be those which invade specific tissues (foliar vs. root/stem rot fungi) or specific stages in the development of the plant (young seedling, mature plant, flowering stage, etc.). The feasibility of artificially combining different cis-acting DNA sequence elements to achieve a given pattern of gene expression in transgenic plants has been demonstrated by Strittmatter, G. and Chua, N. H. (1987, Proc. Natl. Acad. Sci. USA 84:8986-8990).

Although the chitinase coding region in the construct of the present invention is derived from an endochitinase gene from common bean (Phaseolus vulgaris), other structural sequences encoding functionally equivalent chitinase enzymes may also be used. cDNA clones complementary to endochitinase mRNAs have been isolated and characterized from

tobacco (Shinshi, H., Mohnen, D. and Meins, F. (1987) Proc. Natl. Acad. Sci. USA 84:89-93) and potato (Gaynor, J.J. (1988) Nucl. Acids Res. 16:5210). In addition, a chitinase genomic clone has been obtained from tomato (Durand-Tardif, M. (1986) Ph.D. Thesis, Universite de Paris Sud, Centre d'Orsay. 156 pp.). Sequence analysis shows that the cloned tomato, tobacco and potato chitinases share 69%, 73% and 76% homology, respectively, with the chitinase from bean. The presence of chitinase enzyme activity has been demonstrated in many plant species including soybean, sunflower, cotton, corn (Boller, T., Gehri, A., Mauch, F. and Vogeli, U. (1983) Planta 157:22-31), wheat germ (Molano, J., Polacheck, I., Duran, A. and Cabib, E. (1979) J. Biol. Chem. 254:4901-4907), melon (Roby, D., Toppan, A. and Esquerre-Tugaye, M.-T. (1986) Plant Physiol. 81:228-233), barley (Leah, R., Mikkelsen, J.D., Mundy, J. and Svendsen, I. (1987) Carlsberg Res. Commun. 52: 31-37), cucumber (Metraux, J.P. and Staub, T. (1988) Physiol. Molec. Plant Pathol. 33: 1-9) and pea (Mauch, F., Hadwiger, L.A. and Boller, T. (1988) Plant Physiol. 87: 325-333). Using oligonucleotides prepared from strongly conserved regions of the chitinase polypeptide (based upon comparison of the presently available amino acid sequences from bean, tomato, tobacco and potato), one skilled in the art can isolate corresponding chitinase genes from these and other plant sources.

In the 35S-chitinase construct of the present invention, the coding region of bean chitinase is preceded by its cognate 26 amino acid residue signal

peptide. The location of this segment in the bean chitinase 5B gene is indicated in Figure 2 and its primary sequence given below:

NH_2-met-lys-lys-asn-arg-met-met-ile-met-ile-cys-ser-val-gly-val-val-trp-met-leu-leu-val-gly-gly-ser-tyr-gly-COOH

This amino acid sequence is consistent with the tripartite structure found in presequences belonging to the "hydrophobic" group of signal sequences (Verner, K. and Schatz, G. (1988) Science 241:1307-1313). A similar tripartite organization can be found in the signal sequences of other bean proteins destined for localization in the vacuoles of the plant (Doyle J.J., Schuler, M.A., Godette. W.D., Zenger, V. Beachy, R.N. and Slightom, J.L. (1986) J. Biol. Chem. 261:9228-9238; Hoffman, L.M. and Donaldson, D.D. (1985) EMBO J. 4:883-889). Signal sequences of vacuolar proteins in other plants have also been reported (Graham, J.S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. and Ryan, C.A. (1985) J. Biol. Chem. 260:6555-6560; Cleveland, T.E., Thornburg, R.W. and Ryan, C.A. (1987) Plant Mol. Biol. 8:199-207).

Transgenic plants harboring the chimeric 35S-chitinase gene of the present invention are found (Callis, J. Fromm, M. and Walbot, V. (1987) Genes and Development, 1:1183-1200) to correctly and efficiently process the bean chitinase precursor to the mature form of the enzyme. Efficient recognition and cleavage of the bean signal peptide in the heterologous plant background is indicative of its translocation to the central vacuole of the plant cell. In the case of the bean lectin, phytohemagglutinin, correct targeting of this protein

to the vacuoles of yeast has been demonstrated (Tague, B.W. and Chrispeels, M.J. (1987) J. Cell. Biol. 105: 1971-1979). Moreover, fusion of the N-terminal region of the phytohemagglutinin polypeptide to the C-terminal portion of yeast invertase results in re-routing of the normally secreted enzyme to the yeast vacuolar compartment (Tague, B.W. and Christpeels, M.J. (1988) Plant Physiol. 86 (Suppl.):84). While the precise molecular mechanisms involved in the targeting of proteins to different compartments of the cell remain an area of intense research, the above experiments suggest that in the case of chitinase, channelling of the protein to the vacuole may also be achieved by combining the coding region of the mature protein with an appropriate signal sequence derived from other vacuolar-localized proteins. Similarly it should also be possible to target a chitinase enzyme to the intercellular spaces of a plant by combining the coding region of the mature enzyme with a signal sequence derived from a secreted protein such as α-amylase (Chandler, P.M., Zwar, J.A., Jacobsen, J.V., Higgins, T.J.V. and Inglis, A.S. (1984) Plant Mol. Biol. 3:407-418).

Generation of transgenic plants

The chimeric gene of the present invention can be used in transformation experiments to obtain plants exhibiting increased resistance to plant pathogenic fungi. Nucleic acids can generally be introduced into plant protoplasts, with or without the aid of electroporation, polyethylene glycol or other processes known to alter membrane permeability. Nucleic acid constructs can also be

introduced into plants using vectors comprising part of the Ti- or Ri- plasmids, a plant virus or an autonomously replicating sequence. Nucleic acid constructs can also be introduced into plants directly by microinjection or bombardment with DNA-coated microprojectiles into various plant parts. One preferred means of introducing a nucleic acid fragment into plant cells involves the use of Agrobacterium tumefaciens containing the nucleic acid fragment between T-DNA borders either on a disarmed Ti-plasmid (that is, a Ti-plasmid from which the genes for tumorigenicity have been deleted) or in a binary vector in trans to a disarmed Ti-plasmid. The agrobacterium can be used to transform plants by inoculation of tissue explants, such as stems or leaf discs, or by co-cultivation with plant protoplasts. Another preferred means of introducing the present nucleic acid fragment comprises direct introduction of the fragment or a vector containing the construct into plant protoplasts or cells.

The nucleic acid construct of the invention can be used to transform protoplasts or cell cultures from a wide range of higher plant species to form plant tissue cultures of the present invention. These species include the dicotyledonous plants tobacco, petunia, cotton, sugarbeet, potato, tomato, sunflower, soybean, Brassica species and poplars; and the monocotyledonous plants corn, wheat, rice, yam, Lolium multiflorum and Asparagus officinalis. It is expected that all protoplast-derived plant cell lines can be stably transformed with the fragments of the invention.

The nucleic acid fragments of the invention can also be introduced into plant cells with subsequent

formation of transformed plants of the present invention. Transformation of whole plants is accomplished in plants whose cells can be transformed by foreign genes at a stage that can be used to regenerate the whole plant. Transformed plants can be monocotyledonous and dicotyledonous plants. Preferably, the transformed plants are selected from the group consisting of tobacco, petunia, cotton, sugarbeet, canola, potato, tomato, sunflower, carrot, celery, flax, alfalfa, lettuce, cabbage, cucumber, pepper, bean, soybean, Brassica species, poplars, clover, sugarcane, barley, oats, rice and millet; see "Handbook of Plant Cell Culture" Vols. 1-4, Evans, D. A. et al., Sharp, et al., and Ammirato et al., respectively, MacMillan, N. Y. (1983,84,86). The range of crop species in which foreign genes can be introduced is expected to increase rapidly as tissue culture and transformation methods improve and as selectable markers become available.

The cointegrate Ti plasmids containing chimeric chitinase genes were introduced into tobacco by leaf disc transformation. All manipulations of sterile media and plant materials were done in a laminar flow hood, under suitable containment. Plant growth and plant cell cultures were carried out at 27°C.

Healthy, unblemished leaves (4-6 inches in length) from 4-6 week old tobacco plants, grown in a growth chamber, were surface sterilized by immersion in a solution containing 10% commercial bleach (Clorox) and 0.1% sodium dodecyl sulfate (SDS). After 30 minutes of gentle stirring, the solution was poured off and the leaves were rinsed 3 times with sterile water, and then gently shaken to remove excess water. Leaf discs were made by punching 6mm circles with a sterile paper punch.

Cultures of Agrobacterium cells containing the cointegrate plasmid were grown in 5 ml YEB + 100 µg/ml kanamycin (Table I) at 28°C for 16 hr. The cells were collected by centrifugation in an SS-34 rotor at 8,000 rpm for 20 minutes at 22°C, washed with 5 ml YEB, and finally resuspended in 10 ml YEB broth. Approximately 50 leaf discs were briefly submerged in the bacterial suspension and then transferred to petri dishes containing shoot inducing medium (CN) (Table II). The dishes were sealed with parafilm and incubated under mixed fluorescent and "Gro and Sho" plant lights (General Electric) for 2-3 days in a culture room maintained at approximately 25°C.

To rid the leaf disks of Agrobacterium and to select for the growth of transformed tobacco cells, the leaf disks were transferred to fresh CN medium containing 500 mg/l cefotaxime and 100 mg/l kanamycin. Cefotaxime was kept as a frozen 100 mg/ml stock solution and added aseptically (filter sterilized through a 0.45 µm filter) to the media after autoclaving. A fresh kanamycin stock (50 mg/ml) was made for each use and was filter sterilized into the autoclaved media.

Leaf disks were incubated under the growth conditions described above for 3 weeks and then transferred to fresh media of the same composition for an additional 1-2 weeks.

Shoots developing on medium containing kanamycin were excised with a sterile scalpel and planted in root induction medium (A) (Table II) containing 100 mg/l kanamycin. Root formation on selective and non-selective media was recorded within 3 weeks.

Within 2 weeks of planting, small leaves were removed from excised shoots to determine levels of resistance to kanamycin in a callus induction assay on selective media. To induce callus formation, small leaves were excised and cut into several sections with a scalpel and plated on callus induction medium (B) (Table II) containing 50 mg/l kanamycin. Callus growth on selective and non-selective media was recorded within 3 weeks.

The results indicated that transformation of tobacco had been achieved with the GV35CHN strains based on production of kanamycin resistant callus.

Twenty-four kanamycin resistant transgenic tobacco plants were selected and analyzed further for expression of the bean chitinase polypeptide. 3-4 leaves (500-1000 mg fresh wt) were excised from tobacco seedlings and homogenized in a small amount of buffer containing 50 mM HEPES, pH 6.8, 5% mercaptoethanol, 10 mM diethyldithiocarbamic acid (to inhibit polyphenol oxidase activty) and 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 1 mM ∈-amino caproic acid (to inhibit proteases). The homogenized tissue was filtered through two layers of cheesecloth and the filtrate centrifuged at 20,000 rpm x 30 minutes to remove membranes. Soluble proteins were precipitated by the addition 1/10 volume of 100% trichloroacetic acid (TCA). After incubation on ice for 30 minutes, the precipitated protein was collected by centrifugation in a microfuge, and washed twice with 80% acetone. After the final wash, the protein was dispersed in 80% acetone by sonication. The protein concentration of the acetone suspension was determined spectrophotometrically using the Bio-Rad Dye Reagent (Bio-Rad, Richmond, CA).

To determine chitinase levels in the transgenic plants, 20-50 µg of protein, suspended in acetone, was collected by centrifugation and dispersed in 10 µl of 100 mM sodium carbonate, 100 mM dithiothreitol. An equal volume of a 5% SDS, 30% sucrose, 0.1% bromophenol blue solution was then added and the sample heated to 100°C for 2 minutes. The solubilized protein was then subjected to SDS-polyacrylamide gel electrophoresis on a 7.5-15% gradient of acrylamide. After electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose membrane. The immobilized bean chitinase polypeptide was detected on the nitrocellulose by reaction with specific antibodies raised against purified bean chitinase. The specific antigen-antibody complex was visualized using an alkaline phosphatase conjugated goat anti-rabbit IgG. Sites of antigen localization are visualized as a dark blue band resulting from the activity of alkaline phosphatase on color development substrates (Promega, Madison, WI).

The results of this immunological analysis indicated that: (1) leaf samples of the twenty-four assayed kanamycin resistant tobacco plants were found to express the bean chitinase protein; (2) the molecular weight of the immunodetected chitinase protein in tobacco plants was identical to that in bean plants indicating that the precursor polypeptide was correctly processed in the heterologous system. In bean plants, chitinase is synthesized by a co-translational mechanism on membrane bound ribosomes as a precursor polypeptide of ~32 kd. The precursor polypeptide is processed to a mature size of 30 kd during the course of its

transport to the vacuole. The identification of a 30 kd bean polypeptide in individual transgenic tobacco plants is evidence that (1) the signal peptide is cleaved in the heterologous system, (2) the enzyme has been transported to the vacuole, and (3) the bean chitinase polypeptide is expressed constitutively.

Transgenic tobacco plants expressing the bean chitinase polypeptide were found to contain a 1.5-2.5-fold increase in the level of chitinase enzyme activity when compared to control tobacco.

Fungal Resistance Studies - Utility of Invention

Rhizoctonia solani, is an endemic chitinous soil fungus which infects many plant species, including corn and soybeans, and produces severe stem and root rotting symptoms. Although Rhizoctonia rarely kills the plants it infects, seed planted in heavily infested fields have problems with standability and early season growth. This disease is especially severe on oilseed rape grown in Canada. Infection by Rhizoctonia generally results in stunting and an overall reduction in seed yields. Rhizoctonia is a very adaptable organism which can survive in dry soils, wet soils, warm temperatures and cold temperatures. It is a very common soil inhabitant and feeds not only on live plants but also on crop residue.

In the past, symptoms of Rhizoctonia infection have largely been attributed to poor seed quality, herbicide damage and low fertility and not to the presence of the fungus (Kirby, W. (1987) Seed Trade News, p. 28-30). Research has shown that the severity of disease caused by Rhizoctonia can be

augmented by herbicide treatment. Herbicides tend to disrupt the growing point leading to increased absorption of water causing the roots and stems to crack. Breakage of the external tissues in these areas makes it easier for the pathogen to gain entry into the plant. Although the effects of fungi like Rhizoctonia and Fusarium can be minimized by treatment with fungicide, either as a seed treatment or as a soil fumigant, current issues concerning the environmental safety of these chemicals may preclude their future use.

Damping-off, seedling blight and brown girdling root-rot are important diseases of young seedlings. In the canola growing areas of the Peace River region of northwestern Alberta, partial to nearly complete loss of plant stands and an 80-100% infection of established stands have been reported (Davidson, J.G.N. (1977) in "Rapeseed Production on the Peace River region of Alberta" NGR-77-7. Agric. Can. Res. Stn., Beaverlodge, Alberta. 37 pp.). In 1983 and 1984, the estimated average yield loss due to root rot was 36 and 23%, respectively. The organism most frequently associated with the root rot complex of canola is Rhizoctonia solani; it is the only organism isolated from diseased canola plants that is capable of inducing symptoms on artificially inoculated seedlings that are similar to the symptoms observed on seedlings damped-off in the field (Gugel, R.K. et al. (1987) Can. J. Plant Path. 9:119-128).

In the present invention, transgenic plants have been obtained that are resistant to Rhizoctonia solani. Resistance is due to the presence of a modified chitinase gene of the present

invention which allows over-expression, of a bean chitinase enzyme. When transgenic tobacco and oilseed rape plants, containing the modified chitinase gene, are grown in soil inoculated with R. solani, the survival rate is enhanced when compared to that of control plants lacking the modified chitinase gene. The increased survival rate of the transgenic plants is dependent upon the concentration of R. solani inoculum applied to the soil. In a quantitative assay, in which 12-14 day-old transgenic tobacco plants are transplanted into soil infested with increasing amounts of R. solani, near normal root growth is observed in plants containing the modified chitinase gene, while control plants are found to contain significant root damage as a consequence of fungal attack. At the highest inoculum tested (4 ml/pint of soil), control plants suffer as much as a 50% loss in root fresh weight. Under these same conditions, five independently isolated transgenic tobacco plants show an average 10% reduction in root fresh weight. Three of the five plants tested showed only a 4% reduction in root mass when compared to uninoculated plants. These results, consistant with the results of survival tests, demonstrate that transgenic plants exhibit an increased resistance to infection by R. solani when production of chitinase is controlled by a constitutive promoter, in this case, the 35S promoter of cauliflower mosaic virus. The resistant phenotype is further manifested by a delay in progression of the disease with time. This affords the young seedlings an opportunity to continue to grow and develop long enough to acquire their own natural resistance to damping-off diseases.

While Rhizoctonia is a soil-borne pathogen which produces severe root and stem rotting disease, the utility of the present invention is not limited to R. solani. Essentially all fungi, except the oomycetes, contain chitin in their cell walls and are potential targets. Transgenic tobacco plants of the present invention also exhibit increased resistance to the foliar pathogen Botrytis cinerea, a sclerotinaceous ascomycete, commonly referred to as grey mold. This pathogen is responsible for significant post-harvest deterioration of fruits and vegetables, especially strawberries and grapes. Transgenic plants which are inoculated with conidia of B. cinerea were found to exhibit a reduction in the number and size of the lesions produced on young leaves. Three of the five transgenic plants tested, #329, #230 and #238 exhibited an average 30%, 23% and 60% reduction, respectively, in lesion size when compared to control tobacco plants inoculated under the same conditions. Two additional transformants which showed no reduction in fungal damage were found to contain 2- to 4-fold lower levels of the bean chitinase polypeptide in their leaves when compared to other transformants. While the CaMV 35S promoter is a constitutive promoter, the absolute levels and tissue-specificity of genes expressed under the control of this promoter can also be influenced by the environment surrounding the chromosomal insertion site. As stated previously, it may be possible to use alternative leaf specific promoters, such as the rbcS or Cab promoters, to enhance the levels of chitinase in leaves of transgenic plants in order to combat more effectively infection by foliar pathogens.

Transformation of tomato plants with the chimeric gene of the present invention may be used to provide protection against such tomato pathogens as Alternaria, Botrytis, Colletotrichum, Rhizoctonia, Sclerotium, Selerotinia, and Fusarium. Additionally, the stable introduction of the chimeric gene into rice may be of commercial value against the causal agent of rice sheath blight, Rhizoctonia oryzae. In oilseed rape, the potential targets of commercial value include white mold (Sclerotinia), blackleg (Phoma) and brown-girdling root rot (Rhizoctonia solani). Resistance to these pathogens may be enhanced further by choosing the appropriate promoter, transcription stimulator, and termination signals fused to the coding region of a higher plant chitinase gene in order to create transgenic plants with optimum resistance to either a broad range of fungal pathogens or to specific fungal pathogens, whether foliar or root/stem pathogens.

The present invention is further defined in the following EXAMPLES, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these EXAMPLES, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these EXAMPLES, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE 1

Construction of pK35CHN

The plasmid pCH35Δ6 provided a convenient starting point in the construction of pK35CHN. pCH35Δ6 consists of a deleted chitinase gene comprised of approximately 600 bp of 5' flanking DNA, the 981 bp chitinase open reading frame and approximately 1700 bp of 3' flanking DNA contained within the plasmid vector pEMBL8+. Plasmid DNA was isolated from E. coli JM 101 cells harboring pCH35Δ6 according to the procedure of Birnboim and Doly [Nucleic Acids Research (1979) 7:1513] and purified by CsCl/ethidium bromide density gradient centrifugation. 100 µg of purified plasmid DNA was digested to completion with 200 units HindIII in 300 µl 25 mM Tris-HCl, pH 7.8, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 100 µg/ml BSA (medium salt buffer) at 37°C. The linearized DNA was purified by phenol/chloroform and ether extraction and concentrated by precipitation with ethanol. The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the concentration determined by measuring the absorbance at 260 nm assuming an extinction coefficient of 20 cm^2/mg.

50 µg of linearized pCH35Δ6 DNA was incubated at 30°C with 2.5 units of nuclease Bal 31 in 500 µl of buffer containing 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 12 mM $MgCl_2$, 12 mM $CaCl_2$, 1 mM EDTA and 250 µg/ml bovine serum albumin (BSA). 50 µl aliquots of the digestion mixture were removed at 3, 6, 8, 10, 11, 12, 13, 14, 15 and 16 minutes and the Bal 31 digestion quenched by the addition of EGTA to 20 mM final concentration. Following dilution of 5 ul of each time point with 8.5 ul of H_2O and 1.5 µl of a

low salt buffer (250 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 10 mM DTT and 1 mg/ml BSA), 5 units of the restriction enzyme HindII were added and the samples were incubated at 37°C for 2 hours. The progress of the Bal 31 digestion was assessed by agarose gel electrophoresis of the HindII digested samples. From this analysis it was determined that in the sample which was digested for 12 minutes with Bal 31, an average of 600 bp was removed from the 5' end of the gene. The DNA of this sample was purified by phenol/chloroform and ether extraction followed by precipitation with ethanol. The DNA was repaired in an end-filling reaction using 4 units of the Klenow fragment of E. coli DNA polymerase I in 50 µl 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT, 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP and 50 µg/ml BSA. The reaction was incubated at 22°C for 30 minutes and terminated by heating to 70°C for 5 minutes. 2 µg of the blunt-ended DNA was ligated to 0.75 µg phosphorylated HindIII linkers as described in T. Maniatis, E. F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N. Y. (1982). The ligation was allowed to proceed at 15°C for 16 hours after which time the DNA was purified by phenol/chloroform extraction and precipitation with ethanol. The DNA was centrifuged, washed with 80% ethanol and dissolved in 37 µl H_2O. The DNA was digested with excess HindIII (80 units) in 50 µl medium salt buffer at 37°C for 4 hours. After this period of time, the salt concentration was increased to 100 mM NaCl and 20 units of BglII were added. The reaction was incubated at 37°C for an additional 2 hours. The digested DNA was concentrated by ethanol

precipitation and subjected to electrophoresis on a 0.8% low melting point agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2). When the bromophenol blue dye marker had migrated three quarters of the way into the gel, electrophoresis was halted, and the gel was stained in 1 µg/ml ethidium bromide for 20 minutes and destained in H_2O for 10 minutes. After visualization under long wave UV light, the HindIII-BglII chitinase fragment was excised from the gel and the agarose melted at 68°C. The DNA was ligated to 0.48 µg of HindIII + BamHI digested pEMBL8+ in 170 µl 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP containing 3 units T4 DNA ligase. After incubation at 12.5°C for 16 hours, 10 µl of the ligation mix was used to transform E. coli strain JM 101. Transformants were selected by plating on LB agar containing 100 µg/ml ampicillin.

Single stranded DNA was isolated from individual transformants by the method of Dente et al (Nucleic Acids Research (1983) 11:1645). Single colonies were inoculated into 1.5 ml LB broth containing 100 µg/ml ampicillin and 1.7×10^8 IR1 phage/ml. The cultures were allowed to grow at 37°C overnight after which time they were centrifuged in an Eppendorf microfuge at maximum speed for 7 minutes. One ml of the supernatant was mixed with 250 µl of 20% polyethylene glycol, 2.5 M NaCl and the single stranded DNA containing phage were precipitated at 4°C for 30 minutes. The samples were centrifuged for 10 minutes at 4°C, resuspended in 120 µl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.3 M sodium acetate and 0.0025% bromophenol blue, and extracted with 100 µl phenol. The aqueous phase was

extracted twice with 1.4 ml ether and the single stranded DNA precipitated by the addition of 300 µl of ethanol. After 1 hour incubation at -70°C, the DNA was collected by centrifugation, washed with 80% ethanol and dissolved in 25 µl TE, pH 8.0.

Eight µl single stranded DNA was combined with 2.5 ng M13 sequencing primer in 10.5 µl 14.3 mM Tris-HCl, pH 8.0, 7.1 mM MgCl. The samples were placed in a water bath initially set at 75°C and the template and primer were allowed to anneal by slow cooling of the samples below 35°C. The annealed DNA was sequenced by the chain termination procedure of Sanger et al (Proc. Natl. Acad. Sci. USA (1977) 74:5463) and the products of the sequencing reactions were resolved on buffer gradient sequencing gels (Biggin, M. D., Gibson, T. J. and Hong, G. F. [1983] Proc. Natl. Acad. Sci. USA 80:3963). From this nucleotide sequence analysis, it was determined that the 5' ends of two clones (641 and 695) were located 21 and 3 bp upstream respectively of the ATG initiation codon.

Plasmid DNA was isolated from clones 641 and 695 by a mini-prep version of the alkaline-SDS lysis procedure of Birnboim and Doly (Nucleic Acids Res. 7:1513). Cultures were grown in 10 ml LB broth containing 100 µg/ml ampicillin at 37°C overnight. The cells were harvested, resuspended in 400 µl 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose and 5 mg/ml lysozyme and incubated at room temperature for 5 minutes. After this time, 800 µl of 0.2 N NaOH, 1% SDS was added, the samples were mixed gently and then incubated on ice for 5 minutes. 600 µl of 3M potassium acetate, pH 4.8, was added and incubation continued for 5 minutes on ice. The samples were

centrifuged at 8000 rpm (SS-34) for 10 minutes to remove precipitated protein, high molecular weight RNA and chromosomal DNA and the supernatant extracted twice with an equal volume of phenol/chloroform. Plasmid DNA was precipitated at room temperature for 2 minutes by the addition of 2.5 volumes of absolute ethanol. The precipitated DNA was collected by centrifugation, washed with 80% ethanol and dissolved in 200 µl TE buffer containing 20 µg/ml ribonuclease.

Eight µl of each DNA sample was digested with 6 units XhoII in 30 µl 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.01% Triton X-100 and 100 µg/ml BSA at 37°C for 2 hours. After this time, 5 µl 250 mM Tris-HCl, pH 7.8, 500 mM NaCl, 100 mM $MgCl_2$, 10 mM DTT and 1 mg/ml BSA and 30 units HindIII were added, the sample volumes adjusted to 50 µl with H_2O and incubation continued at 37°C for 2 additional hours. One-fifth volume of gel loading buffer (25% Ficoll, 0.25% bromophenol blue and 0.25% xylene cyanol) was added to each sample and 15 µl of the 641 and 695 digests were run on a 0.75% low melting agarose gel in TAE buffer. Following electrophoresis, the gel was stained as indicated above and the 1.5 kb HindIII-XhoII fragments excised.

The plasmid, pK35CAT, described previously, was digested with 30 units each BamHI and HindIII in 60 µl medium salt buffer for 2 hours at 37°C to remove the chloramphenicol acetyl transferase coding fragment. Two 0.5 µg aliquots of the digested plasmid DNA were electrophoresed on a 0.75% low melting point agarose gel. The vector bands containing the 35S promoter and the NOS 3' fragment

in pBR322 were excised, combined with the 1.5 kb fragments of clones 641 and 695 and ligated essentially as described above. A 10 µl aliquot of each ligation mixture was used to transform E. coli strain HB101 cells. Individual transformants were characterized by restriction enzyme digestion of plasmid mini-prep DNA. Transformants which contained the correct fragments generated by SphI digestion were designated p35CHN641 or p35CHN695 depending upon the source of the chitinase fragment.

p35CHN641 and p35CHN695 plasmid DNA was isolated by the alkaline-SDS lysis procedure and purified by CsCl/ethidium bromide density gradient centrifugation. 10 µg of each plasmid was digested with 20 units EcoRI in 50 µl 25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 100 µg/ml BSA containing 5 units calf intestinal alkaline phosphatase. After 1.5 hours at 37°C, 5 µl 1M Tris-HCl, pH 8.8 and 5 units more phosphatase were added and the samples incubated at 55°C for 30 minutes. After this time, EDTA was added to a final concentration of 10 mM and the samples heated to 70°C for 5 minutes. The digested DNAs were purified by phenol/chloroform and ether extraction, followed by precipitation with ethanol. A 0.4 µg aliquot of each vector was combined with 0.1 µg of an EcoRI fragment bearing an NPTI and a chimeric NOS/NPTII/OCS gene in 25 µl ligation buffer containing 1 unit T4 DNA ligase. After 16 hours at 12.5°C, a 5 µl aliquot of each ligation was used to transform E. coli strain HB101 cells. Transformants were selected on LB agar containing 100 µg/ml kanamycin. Plasmid DNA was isolated from individual transformants by a mini-prep version of the

procedure of Birnboim and Doly. The presence of the EcoRI fragment was confirmed by restriction enzyme digestion of the isolated DNA. Digestion with the restriction enzyme HindIII additionally permitted determination of the orientation of the inserted kanamycin resistance marker fragment.

E. coli strains HB101 carrying the plasmids pK35CHN641 and pK35CHN695 were deposited September 23, 1988 in American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A under the terms of the Budapest Treaty. The deposit identification numbers are ATCC67811 and 67812, respectively.

EXAMPLE 2

Agrobacterium mediated transformation of tobacco

The recombinant DNA construct described in Example 1 was transformed into tobacco by Agrobacterium tumefaciens infection of tobacco leaf discs. Primary transformants were analyzed to demonstrate constitutive expression of the bean chitinase polypeptide in tobacco. Progeny of the transformants were also analyzed to demonstrate resistance to fungal infection and inheritance of the inserted DNA construct. Standard aseptic techniques for the manipulation of sterile media and axenic plant/ bacterial cultures were followed, including the use of a laminar flow hood for all transfers. The plasmids pK35CHN641 and pK35CHN695 were introduced into Agrobacterium tumefaciens strain GV 3850 (Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M. and Schell, J. (1983) EMBO Journal 2:2143) by conjugation using the three way mating method of Ruvkin, G. and Ausubel,

F. M. (Nature [1981] 289:85). E. coli HB 101 containing the pK35CHN plasmids and E. coli HB 101 containing the mobilization plasmid pRK2013 [Figurski, D. and Helinski, D. R. (1979) Proc. Natl. Acad. Sci. USA 76:1648 (ATCC 37159)] were separately inoculated into 3 ml LB broth containing 100 µg/ml kanamycin and allowed to grow at 37°C overnight. A 3 ml liquid culture of A.tumefaciens GV 3850 was grown overnight in LB broth at 28°C (to avoid curing of the Ti plasmid). The cells were harvested by centrifugation at 3000 rpm for 10 minutes in an SS-34 rotor at 4°C. The cells were washed with 3 ml broth and finally resuspended in 3 ml LB. 100 µl of each type of cells (pK35CHN, pRK2013, GV 3850) were mixed in a sterile Falcon test tube and the entire mixture aseptically applied to a sterile 0.45 micron Millipore filter. After the liquid had filtered through, the filters were transferred to LB agar plates and incubated at 28°C for 20 hours. The filters were transferred to Falcon tubes and the cells washed off the solid support with 0.5 ml 10 mM $MgSO_4$. 5 µl of each sample was spread on selective media (0.22 M Na_2HPO_4, 0.22 M KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.4% sucrose and 1 mg/ml kanamycin) and the plates incubated at 28°C. After 3 days, cointegrates were visible as fairly large, white, opaque colonies on a light lawn of bacteria.

Four cointegrates from each mating were streaked for single colonies on LB agar containing 100 µg/ml of both kanamycin and rifampicin and incubated at 28°C for 2 days. Single colonies were then inoculated into 5 ml LB broth containing 100 µg/ml kanamycin and the cultures allowed to grow at

28°C for 27 hours. Total DNA was isolated from the Agrobacterium samples for Southern blot analysis in order to probe the integrity of the construct which had become integrated into the Ti plasmid by homologous recombination. The cells were harvested by centrifugation at 6000 rpm for 10 minutes in an SS-34 rotor at 4°C. The pellets were resuspended in 100 µl 0.15 M NaCl, 0.1 M EDTA and 25 µl of a fresh solution of lysozyme (2mg/ml) was added. The samples were incubated at 37°C for 30 minutes and then transferred to a dry ice/ethanol bath. After thawing, 125 µl 0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 1% SDS was added and the samples mixed gently by inversion. They were then extracted once with phenol, once with chloroform and the DNA precipitated by the addition of 2.5 volumes of absolute ethanol. The precipitated DNA was dissolved in 50 µl TE buffer, pH 8.0 containing 20 µg/ml ribonuclease and 5 µl of each was digested with 10 units EcoRI and 11 units ClaI in 15 µl medium salt buffer. A second 5 µl aliquot was digested with 10 units HindIII and 11 units ClaI in 15 µl medium salt buffer for 4 hours at 37°C. The restriction enzyme digests were loaded on a 0.8% agarose gel and electrophoresed at 30 volts overnight. The DNA was transferred to a nitrocellolose filter according to the method of Southern, E. (J. Mol. Biol. [1975] 98:503). The DNA blots were then probed with nick translated EcoRI insert of pCH18 in order to verify the integrity of the chimeric chitinase gene.

Potted tobacco plants (for leaf disc infections) were grown in a growth chamber maintained for a 12 hr, 24°C day and for a 12 hr,

20°C night cycle, with approximately 80% relative himidity, under mixed cool white fluorescent and incandescent lights. Tobacco leaf disc infections were carried out essentially by the method of Horsch, R. B., Fry, J. E., Hoffmann, H. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. (1985) Science 227:1229-1231.

Young leaves, not fully expanded and 4-6 inches in length were harvested from 4-6 week old tobacco plants (Nicotiana tabacum cv xanthi). The leaves were surface sterilized for 30 minutes by submerging them in approximately 500 mls of a 10% Clorox, 0.1% SDS solution and then rinsed three times with sterile distilled water. Leaf disks, 6 mm in diameter, were prepared from whole leaves using a sterile paper punch.

Leaf disks were inoculated by submerging them for several minutes in 20 mls of a 1:10 dilution of an overnight culture of Agrobacterium. The culture was started by inoculating 5 mls of YEB broth (Table I) containing 100 µg/ml kanamycin with a single bacterial colony removed from an LB plate (Table I) containing 100 µg/ml rifampicin and 100 µg/ml kanamycin. The culture was grown for 17-20 hours in a 15 ml disposable Falcon tube in a New Brunswick water bath shaker at 28°C.

After inoculation, the leaf discs were placed in petri dishes containing CN agar medium (Table II) and sealed with parafilm. The petri dishes were incubated under mixed fluorescent and "Gro and Sho" plant lights (General Electric) for 3 days in a culture room maintained at approximately 25°C.

To rid the leaf discs of Agrobacterium and to select for growth of transformed tobacco cells, the

leaf discs were transferred to fresh CN medium containing 500 mg/l cefotaxime and 100 mg/l kanamycin. Cefotaxime was kept as a frozen 100 mg/ml stock solution and added aseptically (filter sterilized through a 0.45 µm filter) to the media after autoclaving. A fresh kanamycin stock (50 mg/ml) was made for each use and was filter sterilized into autoclaved media. Leaf discs were incubated under the growth conditions described above for 3 weeks and then transferred to fresh medium of the same composition.

Approximately 1-2 weeks later, shoots developing on explants grown on kanamycin-containing media were excised using a sterile scalpel and planted in medium A containing 100 mg/l kanamycin (Table II). Root formation and callus induction on selective media was recorded within 3 weeks (Table III). Shoots which rooted in kanamycin were transferred to soil and grown in a growth chamber as described above. After 3-5 weeks, but before flowering had occurred, leaf tissue was excised and used for immunological identification of the bean chitinase polypeptide. This was accomplished by Western blot analysis (Burnette, W. N. Anal. Biochem. 112:195) and was performed according to the following protocol:

1. Grind approximately 500-1000 mg (fresh wt.) of leaf tissue in 1 ml of homogenization buffer containing 50 mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid), pH 6.8, 5% B-mercapto-ethanol, 10 mM diethyldithiocarbamic acid, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine and 1 mM ∈-amino-n-caproic acid, in a mortar and pestle.

2. Filter through 2 layers of cheesecloth and centrifuge filtrate at 2,000 rpm in a Sorvall SS34 rotor for 30 minutes to remove membranes.
3. Add 1/10 volume of 100% TCA to precipitate protein. After 15-30 minutes on ice, centrifuge at 12,000 x g in a microfuge to pellet protein.
4. Disperse pellet in 500 µl of 80% acetone by sonication and centrifuge again to collect protein.
5. Repeat step 4.
6. Remove an aliquot of the protein suspension and centrifuge to pellet protein. Dissolve protein in 50 µl 0.1 N NaOH and neutralize with 0.1 N HCl. Determine the protein concentration using the Bradford reagent according to the manufacturer's specifications (Bio-Rad, Richmond, CA).
7. Remove an aliquot of the acetone suspension containing at least 20 µg of protein and collect the protein pellet by centrifugation, discard supernatant.
8. Disperse protein in 10 µl of 0.1M sodium carbonate, 0.1 M dithiothreitol (DTT). Add an equal volume of 5% SDS, 30% sucrose, 0.1% bromophenol blue. Boil samples for 90 seconds.
9. Separate proteins by electrophoresis on polyacrylamide gels consisting of either a 7.5-15% gradient of acrylamide or 12% acrylamide prepared as described by Piccioni, R., Bellemare, G. and Chua, N-H. (1982) in "Methods in Chloroplast Molecular Biology", pg 985ff, Elsevier Biomedical Press, New York, N.H.

10. Transfer proteins from the acrylamide gel to nitrocellulose filters (BA45, Schleicher and Schuell) using an E-C Electroblot® Electrophoretic Transfer System (or equivalent) according to manufacturer's specifications (E-C Apparatus Corp., St. Petersburg, FL).
11. Wash nitrocellulose filter in 50 mls TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20, 0.02% sodium azide). Discard wash.
12. Incubate filter in 50 mls TBST + 1% bovine serum albumin (Promega, Madison, WI) for 30 minutes with gentle shaking.
13. Incubate filter for 30 minutes with 50 mls of TBST containing a 1:20,000 dilution of rabbit anti-chitinase IgG. Antibodies were raised in rabbits by injecting a mixture of gel purified chitinase and Freund's complete adjuvant (Difco) into the subscapular space of a New Zealand white rabbit.
14. Wash three times for 10 minutes each with 50 mls TBST.
15. Incubate filter with 50 mls TBST containing a 1:7500 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG (Promega, Madison, WI).
16. Repeat step 14.
17. Add 50 mls AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 330 µl NBT (nitroblue tetrazolium) and 165 µl BCIP (5-bromo-4-chloro-3-indolyl phosphate, Promega, Madison, WI, catalogue No. W3930). Incubate until blue color appears verifying the presence of the bean chitinase polypeptide.

18. Stop the reaction by rinsing extensively in water.

In all, 24 different plants were analyzed by immunological analysis for expression of the modified chitinase gene. Figure 4 shows the results of this experiment for 8 transformants containing the chimeric chitinase gene. These independent transformed lines represent plants containing either plasmid pK35CHN641 or pK35CHN695. No differences were observed in the level of chitinase expression among these plants other than those attributable to the effect of different chromosome insertion sites. To demonstrate that constitutive expression of the bean chitinase polypeptide in transgenic tobacco resulted in increased enzyme levels in uninfected (uninduced) plants, a chitinase enzyme assay was performed on several transgenic lines. These assays were performed on lines which were homozygous for the introduced trait (see below). Samples of roots, stems and leaves from uninfected plants were collected from three independent transformants: #373, #238 and #548 (control). Two to four grams of tissue were extracted in 8 mls of 20 mM phosphate buffer, pH 6.4 by grinding with a motar and pestle. The homogenate was centrifuged for 10 min at 10,000g. The supernatant was concentrated approximately 2- to 3-fold using a commercialy available Centriprep concentrator (Amicon #4206) and the protein concentration determined using the Bradford assay (BioRad).

Chitinase enzyme activity was determined using a radiometric assay which utilized regenerated radioactive chitin as a substrate (Molano, J., Duran, A. and Cabib, E. (1977) Anal. Biochem., 83:648-656).

The reaction mixture consisted of enzyme extract (25 µg protein), 1 mg [^{3}H] chitin, 0.3 mmol sodium azide, 20 mM sodium phosphate (pH 6.5) in a final volume of 0.25 ml. The reaction was stopped after 90 min at 37°C by the addition of 0.25 ml 1M trichloroacetic acid. After centrifugation (1,000g for 5 min), the radioactivity of 0.3 ml of the supernatant was determined by liquid scintilation counting. The results of this analysis, shown in Table IV, demonstrate that transgenic tobacco plants containing the modified chitinase gene exhibit approximately 1.5-2.5-fold increases in the level of chitinase enzyme activity.

TABLE I

BACTERIAL GROWTH MEDIA

RR MEDIUM

Add 7.5 g agar to 440 ml of water, autoclave, and keep at 55°C. Add the following sterile stock solutions:

0.5 ml 1 M $MgSO_4$
0.5 ml 1 M $CaCl_2$
10.0 ml 20% Sucrose
5.0 mls 100 mg/ml Kanamycin
50.0 mls 10 x salts (Na_2HPO_4 . $7H_2O$, 60 g/l; KH_2PO_4, 30g/l; NaCl, 5g/l; NH_4Cl, 10 g/l).

LB MEDIUM

	Per Liter
NaCl	10.0 g
Bacto-Yeast Extract	5.0 g
Bacto-tryptone	10.0 g

Adjust pH to 7.5 with sodium hydroxide

YEB MEDIUM

	Per Liter
Bacto Beef Extract	5.0 g
Bacto Yeast Extract	1.0 g
Peptone	5.0 g
Sucrose	5.0 g
$MgSO_4$. $7H_2O$	0.5 g
Agar (Optional)	15.0 g

MIN A

1. Add 7.5 g agar to 400ml water
2. Make stock:

K_2HPO_4	5.25 g
KH_2PO_4	2.25 g
$(NH_4)_2SO_4$	0.5 g
Sodium Citrate·$2H_2O$	0.25 g
	100 ml

TABLE I (continued)

3. Make $MgSO_4 \cdot 7H_2O$ stock= 20g /100 ml, autoclave
4. Make glucose stock= 20 % solution, autoclave

To make Min A:

Mix (1) and (2)
Add 0.5 ml of (3), 5 ml of (4).

TABLE II

TOBACCO TISSUE CULTURE MEDIA

Callus Induction Medium (B)

1 Package of MS salts (Gibco Murashige Organics Medium) with 3% Sucrose	per liter
1 ml of 1 mg/ml NAA	pH 5.8
0.2 ml of 1 mg/ml BAP	
0.8% agar	

Shoot Induction Medium (CN)

1 package of MS salts with 3% Sucrose	per liter
1 ml of 1 mg/ml NAA	pH 5.8
1 ml of 1 mg/ml BAP	
0.8% agar	

Root Induction Medium (A)

1 package of MS salts (without sucrose)	per liter
10 grams Sucrose	pH 5.8
0.8% agar	

TABLE III

Results of tobacco leaf disc transformation with GV35CHN strains

# Shoots	% Root Induction	% Callus Initiation
GV35CHN (695) 149	22/149	22/149
GV35CHN (641) 140	17/140	24/140
GV35K 50	10/50	17/50

TABLE IV

Results of chitinase assay in transgenic tobacco plants

Plant #	Chitinase activity (cpm/mg protein/min)		
	Roots	Stems	Leaves
373	2.6	2.6	2.7
238	2.3	2.3	2.6
548	1.5	1.3	1.1

EXAMPLE 3

Resistance of transgenic tobacco to Rhizoctonia solani

Trangenic tobacco plants of Example 2 containing the modified chitinase gene were analyzed for resistance to fungal pathogens. Five independent tobacco transformants expressing the modified chitinase gene were chosen for analysis: #230, #235, #238, #329 and #373.

In the first experiment, four replicates each of R1 progeny (a segregating population) derived from transformants #235 and #329, which contained the modified chitinase gene(GV35CHN transformants), were used to assay for fungal resistance. Control plants for these experiments were obtained using the same transformation and regeneration conditons as those used to generate experimental plants except that the Agrobacterium strain used contained the co-integrate plasmid GV35K. Plasmid GV35K is identical to GV35CHN except that it lacks the modified chitinase gene. In subsequent experiments, Projeny of GV35K transformant #548 were used as a control.

Seedlings grown on kanamycin-containing SG media were transferred to sterile soil and covered with plastic to maintain a humid environment. After 1 week the plastic was removed and the plants were transplanted into soil containing two ml of R. solani inoculum per pint of soil. Inoculum for these experiments was prepared by growing R. solani on a sand/cereal medium consisting of 500 ml quartz sand, 40 ml cream of wheat, 40 ml corn meal and 75 ml water. The medium was prepared by placing the corn meal and cream of wheat in a metal mixing bowl with 500 ml quartz sand and mixing thoroughly. The medium was then poured into a wide mouth jar, covered with a glass petri dish top, and autoclaved for 2.5 hours.

Upon removing from the autoclave, the media was shaken to loosen and break up the sand /cereal in order to prevent hardening. This medium is suitable for growing a number of soil organisms including Rhizoctonia, Sclerotinia, Fusarium, and Thielaviopsis.

The number of plants surviving this treatment were recorded approximately one week later (Table V). In this experiment, #235 had the greatest survival rate, while plant #373 was indistinguishable from the control plants. In a second experiment performed as described above, ten replicates of #230, #235, #238, #329, #373 and #548 were assayed for resistance after being transferred to soil containing four ml of inoculum per pint of soil. Survival rates recorded after 16 days post infection show that all of the transgenic plants containing the chimeric gene of the invention display an increased survival when compared to control tobacco plants (Table V). Moreover, when the surviving plants were analyzed further, those containing the modified chitinase gene were found to have near normal root growth while the control plants, lacking the modified chitinase gene, exhibited significant root damage (see Table VI and Figure 5).

In a third experiment, performed as described above, unusually severe disease symptoms were noted on all plants tested. Applicants believe that this was due to inadvertant root damage to the plants caused by transplanting seedlings from solid medium to potting soil which, in this case, provided an advantage to the attacking fungus. This problem of advantitious infection can be avoided by the use of genetically homozygous stocks of the transgenic tobacco lines which can be planted directly in soil.

To identify such lines, R1 seed of primary transformants #548, #230, #235, #238, #329, and #373 were surface sterilized and germinated on SG medium containing 100 mg/L kanamycin, as described above, to select for plants containing the transferred chitinase and kanamycin resistance genes. Seedlings which were able to develop on the kanamycin-containing medium were transferred to soil and allowed to grow to maturity in the greenhouse. As above, bags were placed on individual flowers to permit self-fertilization. Seeds of several plants derived from individual transformants were collected and subjected to segregation analysis by germinating seed on SG medium containing 100 mg/L kanamycin. R1 plants which were originally heterozygous would produce progeny which segregated with a ratio of 3 resistant:1 sensitive. On the other hand, R1 plants which were homozygous would yield 100% kanamycin resistant progeny after self-fertilization. Using this procedure, homozygous seed stock of each transformant were identified for further analysis.

In order to determine more precisly the level of resistance to R. solani conditioned by the modified chitinase gene, a quantitative assay of fungal resistance was developed based on observed differences in root mass of the infected transgenic plants. Seed of homozygous plants to be evaluated for fungal resistance were grown in soil for 14 days and then transplanted into two inch pots and allowed to continue growing for an additonal 9 days. The approximately three week old seedlings were then transplanted into one pint cups of sand/soil (1:3) infested with either 0 , 1, or 4 ml of dry R. solani inoculum per pint. Mechanical injury to the root system was avoided by transplanting the entire root

ball plus growing medium of the 2 inch pot into the pint container. These plants were then allowed to continue growth in the infected soil for an additional 11 days. The extent of the disease on individual transformants was quantitated by comparing the fresh weight of the roots from infected plants with the fresh weight of roots from uninoculated plants. The results from two independent experiments are shown in Table VI and are summarized in Figure 5. Data points represent mean root fresh weight values of 10 plants and demonstrate that control plants suffer from significant reductions in root fresh weight when grown in the presence of increasing Rhizoctonia inoculum. In contrast, transgenic tobacco containing the modified chitinase gene show near normal root growth in the presence of the fungal pathogen.

In a similar experiment, the survival rate of 32 plants derived from one of the transformed lines containing the modified chitinase gene (#373) was compared to that of control tobacco (#548) grown in Rhizoctonia-infested soil. Eighteen day old seedlings were transferred to soil containing 1 ml R. solani inoculum per liter of soil. This level of inoculum results in the killing of approximately 50% wild type tobacco plants. The extent of infection was monitored by recording the number of surviving plants at various intervals during a 20 day period following inoculation. The results from this experiment are shown in Figure 6. When surviving plants are uprooted and examined, the average root dry weight/plant is 50% greater in plants containing the modified chitinase gene (0.01 g compared with 0.005 g for control plants). The results of this

experiment confirm that transgenic plants containing the modified chitinase gene exhibit increased survival rates compared to control plants when grown in the presence of the pathogenic fungus R. solani.

TABLE V

Survival of transgenic tobacco plants in Rhizoctonia solani infested soil

Plant	Number of Plants Surviving Experiment 1	Experiment 2
230	-Nd	90%
235	100%	90%
238	-Nd	90%
329	25%	60%
373	-Nd	80%
548	25%	40%

-Nd = not determined

TABLE VI

Effect of Rhizoctonia solani inoculation on root fresh weight of transgenic tobacco plants

Experiment 1

REP #	#548 0	#548 1 ml	#548 4 ml	#373 0	#373 1 ml	#373 4 ml
1	2.78	2.53	1.17	2.54	2.54	2.82
2	5.03	2.15	0.95	3.29	2.13	2.88
3	6.32	1.76	1.98	2.62	2.74	2.24
4	4.05	4.44	1.06	2.98	3.94	2.62
5	4.65	1.64	3.17	2.59	2.17	2.65
6	3.34	1.49	2.57	3.51	2.16	1.94
7	3.69	1.13	3.75	2.79	3.37	2.06
8	4.54	1.48	1.49	1.89	3.32	1.91
9	4.27	2.75	1.55	2.42	3.27	2.32
10	3.68	4.60	1.10	2.40	2.95	2.69
Mean	4.24	2.5	1.88	2.70	2.86	2.41
% of Control	100	58.96	44.37	100	105.8	89.27
% of Loss	0	41.03	55.6	0	0	10.72

REP #	#230 0	#230 1 ml	#230 4 ml	#238 0	#238 1 ml	#238 4 ml
1	4.36	3.89	3.03	3.85	4.59	1.67
2	3.95	3.32	3.02	4.36	3.61	3.20
3	3.28	3.79	3.54	3.15	3.76	2.70
4	3.30	3.30	2.78	2.93	2.69	3.16
5	5.03	3.42	2.77	4.11	3.53	3.59
6	4.44	4.07	2.51	3.41	3.77	1.86
7	6.10	2.62	1.28	3.41	4.32	3.30
8	2.76	4.45	2.44	4.63	3.76	2.25
9	4.58	2.40	2.68	3.29	2.18	2.24
10	4.79	3.91	3.17	4.45	3.00	3.35
Mean	4.26	3.52	2.72	3.76	3.52	2.73
% of Control	100	82.6	63.91	100	93.57	72.6
% of Loss	0	17.4	36.09	0	6.43	27.4

TABLE VI (continued)

Experiment 2

REP #	#548			#373			329		
	0	1 ml	4 ml	0	1 ml	4 ml	0	1 ml	4 ml
1	1.83	1.87	1.72	3.5	3.86	2.51	1.31	3.76	1.99
2	3.81	1.49	1.81	3.02	2.93	3.31	2.38	3.78	2.00
3	2.50	2.17	1.29	2.50	3.57	2.22	2.10	2.54	2.47
4	3.28	2.28	2.20	3.04	2.20	2.96	3.25	3.04	2.45
5	3.10	2.45	2.34	3.12	2.08	3.05	3.76	2.53	3.08
6	3.04	2.12	1.24	2.95	3.34	2.04	2.53	3.08	2.61
7	3.05	2.33	1.64	2.83	3.65	3.46	2.32	2.09	3.06
8	3.75	2.55	0.18	3.73	3.47	2.78	1.42	3.33	2.52
9	2.64	3.26	1.73	3.68	2.11	2.91	3.40	2.73	3.77
10	3.67	2.09	2.41	3.27	3.19	2.12	4.24	3.12	2.67
Mean	3.05	2.25	1.65	3.16	3.04	2.73	2.77	3.0	2.63
% of Uninoc	100	73.8	54.17	100	96.08	86.47	100	108	95.2
% of Loss	0	26.2	45.82	0	3.91	13.52	0	0	4.8

REP #	#230			#238		
	0	1 ml	4 ml	0	1 ml	4 ml
1	3.20	4.23	3.10	4.2	3.24	3.10
2	3.31	2.34	2.32	1.41	2.53	2.32
3	3.21	4.65	2.11	3.34	1.94	2.11
4	3.14	3.47	2.62	1.88	3.20	2.62
5	3.51	3.21	2.65	2.98	2.97	2.65
6	3.71	2.86	3.60	2.08	3.43	3.60
7	4.17	3.16	3.44	4.22	2.43	3.44
8	2.65	3.33	3.19	3.23	3.74	3.19
9	2.73	3.72	2.18	2.91	2.17	2.18
10	2.95	3.66	2.38	1.18	1.73	2.38
Mean	3.26	3.46	2.75	2.82	2.73	2.75
% of Uninoc	100	106	84.67	100	96.98	97.73
% of Loss	0	0	15.4	0	3.01	2.26

EXAMPLE 4

Resistance of transgenic tobacco plants to Botrytis cinerea

Transgenic tobacco plants of the present invention were analyzed for resistance to the foliar pathogen Botrytis cinerea. In this Example, five independently isolated transgenic tobacco plants (#329, #235, #238, #230, #373) containing the modified chitinase gene of the invention and control plants (#548) lacking the modified chitinase gene, were tested for resistance to the fungal pathogen B. cinerea. This fungal pathogen, commonly referred to as grey mold, is responsible for significant post-harvest losses on fruits and vegetables, especially strawberries and grapes.

The B. cinerea isolate used in this experiment is a Benlate resistant isolate. This isolate was used because it grows faster and sporulates more profusely than Benylate sensitive isolates, however, any publicly available virulent strain of B. cinerea (such as are available from the ATCC) would be useful for this purpose. B. cinerea was grown on Potato Dextrose Yeast Agar (PDYA) containing 20 mg/L benomyl (99.5% a.i.) PDYA was prepared by melting 39 grams potato dextrose agar (Difco Laboratories, Detroit, Michigan) and 5 grams yeast extract (Difco Laboratories) in 900 ml water on a hot plate stirrer. Benlate (40 mg in 100 ml water) was sonicated until a uniform milky solution was obtained and added to the PDA/yeast extract solution. The medium was autoclaved for 20 min and used to pour plates. Plates were stored at 4°C until use. PDYA plates were inoculated aseptically with B. cinerea by streaking with a dilute suspension of spores and incubating at 20°C for 5-7 days in the

dark. Spore suspensions were prepared when plates appeared grey-brown (5-7 days after inoculation) by washing plates with a 1% solution of yeast extract. Spores were removed using a brush and then filtered through a single layer of cheesecloth to remove mycelia. The concentration of spores was determined using a hemocytometer and adjusted to a final concentration of 100,000/ml.

Seedlings, grown on SG medium containing 100 mg/L kanamycin, were transferred to sterile soil and covered with plastic to maintain a humid environment. After 1 week, the plastic was removed. Thirteen days following removal of the plastic cover, plants were inoculated on the youngest fully expanded leaves(usually 3 per plant) with a small drop of liquid containing 100,000 conidia/ml. Dried grape leaves were ground and carefully sprinkled on top of the inoculated leaves to serve as a nutrient source for the developing pathogen. After drying, plants were placed in a dew chamber for four days at 24°C to allow the disease to progress. Disease severity was scored by determining the number and size of the lesions produced on individual leaves. The data from this experiment are summarized in Table VII. As shown in Table VII and Figure 7, three of the five transgenic plants tested, #238, #329 and #230, exhibited an average 60%, 30% and 23% reduction, respectively, in lesion size compared to control tobacco plant #548. The remaining two transformants analyzed showed no reduction in fungal growth, however the level of bean chitinase polypeptide in the leaves of these plants was at least 2- to 4-fold lower than that found in the other transformants analyzed. These results indicate that transgenic

tobacco plants containing the modified chitinase gene of the present invention, have been obtained which exhibit less severe symptoms (smaller average lesion size) when infected with the foliar plant pathogenic fungus B. cinerea.

Together, the data of Examples 3 and 4 demonstrate the utility of this invention for producing transgenic tobacco plants which are resistant to both soil-borne and foliar fungal plant pathogens.

TABLE VII

Effects of Botrytis cinerea infection on transgenic tobacco plants containing the chimeric chitinase gene

Leaf Number	Lesion Size (cm)[1] #548	#329	#235	#238	#230	#373
1	5.0	3.7	5.2	2.5	3.5	5.5
2	6.3	3.5	6.9	1.7	4.7	4.9
3	3.4	2.9	6.6	1.9	3.3	4.1
Average	4.9	3.4	6.2	2.0	3.8	4.8

[1]Data represent averages of 4 replicates of each transformant.

EXAMPLE 5

Generation of transgenic tomato plants

In this example, the modified chitinase gene of Example 1 is carried as a Kpn I fragment on the binary vector pMChAD in Agrobacterium tumefaciens strain LBA4404. This vector was used to introduce the modified chitinase gene into tomato plants by infection of cotyledon explants. An outline of the features of pMChAD are shown in Figure 8 and are described below. pMChAD was assembled from the parent binary vector pZS97. The plasmid pZS97 contains a left border fragment of the octopine Ti plasmid, pTiA6 and a right border fragment derived from pTiAch5 (van den Elzen, P. et al. (1985) Plant Molec. Biol. 5:149). The border fragments delimit the segment of DNA which becomes stably incorporated into the host plant genome during the process of Agrobacterium-mediated transformation. Between the left and right border fragments is positioned the polylinker sequence of pUC18 and a chimeric marker gene (NOS/NPTII/OCS) which specifies kanamycin resistance in plant cells. The amp^r segment provides ampicillin resistance to bacteria harboring this plasmid and the ori segment is required for replication of the plasmid in E. coli. The rep and sta regions, derived from the pVS1 plasmid of Pseudomonas aeruginosa (Itoh, Y. et al. (1984) Plasmid 11:206), are essential for replication and stable maintenance, respectively, of pZS97 and its derivatives in Agrobacterium tumefaciens.

In addition, the plasmid pMChAD also contains a tobacco acetolactate synthase (ALS) gene. This gene consists of the upstream and termination sequences of the SurB allele and the coding region of the SurA

allele containing a proline to alanine mutation at amino acid 197 and a tryptophan to leucine mutation at amino acid 591. These mutations in ALS confer resistance to sulfonylurea herbicides when introduced into plants. Although pMChAD contains both a herbicide resistance and a fungal resistance gene, data of Exampes 2-4 indicate that only the modified chitinase gene of the present invention is responsible for the fungal resistant phenotype observed in transgenic plants harboring this gene. As is well known by those skilled in the art, any number of Agrobacterium based Ti-plasmid vectors would allow efficient transfer and identification of plants containing the modified chitinase gene of the present invention.

Standard aseptic techniques for the manipulation of sterile media, and axenic plant and bacterial cultures were followed, including the use of a laminar flow hood for all transfers.

Seeds of tomato (Lycopersicon esculentum var. Bonnie Best) were surface sterilized for 30 minutes in a 10% Clorox, 0.1% SDS solution and rinsed 3 times with sterile deionized water. The seeds were planted in Magenta boxes (Magenta Corp.) containing 100 ml of OMS agar medium (Table VIII) and germinated under mixed fluorescent and "Gro and Sho" plant lights (General Electric) in a culture room maintained at approximately 25°C. Cotyledons from 10-15 day old seedlings were used for the Agrobacterium inoculation.

Cotyledons were wounded by removing approximately 2 mm of tissue from each end of the cotyledon with a sterile scalpel. Wounded cotyledons were planted in petri dishes on CTM agar medium (Table VII) either with or without 75 μM acetosyringone (Aldrich Chemical Co.).

In preparation for the cotyledon inoculation, a single colony of Agrobacterium from a Min A (Table IV) agar plate containing 100 µg/ml carbenicillin was inoculated into a flask containing 30 ml of Min A broth and grown for 2 days at 28°C in a New Brunswick shaker incubator. On the morning of the transformation experiment, the bacterial culture was diluted with sterile Min A broth to an OD_{650} of 0.1 and allowed to grow to an OD_{650} of 0.2 under the same growth conditions. This culture was then used undiluted for the transformation experiment.

CTM agar plates (Table VIII) containing the cotyledon explants were flooded with 5 ml of the bacterial suspension for approximately 5 minutes before removal of the solution. The plates were then secured with Time Tape (Shamrock Scientific Specialty Co.) on two sides of the dish and incubated for two days under mixed fluorescent and "Gro and Sho" plant lights at approximately 25°C for two days.

To rid the plant cultures of Agrobacterium and to select for the growth of transformed tomato cells, the cotyledon explants were transferred to fresh CTM medium containing 500 mg/liter cefotaxime and 50 mg/liter kanamycin, respectively, and incubated under the same culture conditions for approximately 3 weeks. After this period of time, the cotyledons were transferred to fresh CTM medium containing the same selective agents as above but with 1/10 the zeatin concentration.

After approximately 2-4 weeks, shoots developing on kanamycin-selected cotyledons were excised and planted in OMS media (Table VIII) containing 500 mg/liter cefotaxime and 100 mg/liter kanamycin sulfate. Table IX summarizes the results

of the transformation experiment. Tomato shoots which developed roots in this medium after approximately 2-3 weeks were transferred to soil in 8 inch pots and covered with plastic bags. The plants were grown under mixed fluorescent and incandescent lights with a 12 hour, 24°C day; 12 hour 20°C night cycle, for one week before removing the plastic bags. The plants were then grown in a greenhouse and leaf tissue assayed for the presence of the bean chitinase polypeptide by Western blot analysis as described in EXAMPLE 2. Figure 9 shows the results of this analysis for three of the four plants transformed with the binary vector pMChAD.

TABLE VIII

Tomato Tissue Culture Medium

CTM Medium

1 pkg MS salts (Gibco)
1 ml B5 vitamins (per 100 ml: Nicotinic Acid 100 mg, thiamine hydrochloride 1000 mg, pyridoxine hydrochloride 100 mg, M-inositol 10,000 mg)
3 mM MES
3% glucose
0.7% agar
pH 5.7
Autoclave and add 1 ml 1 mg/ml zeatin stock

OMS Medium

1 pkg MS salts (Gibco)
1 ml B5 vitamins (see above)
3 mM MES
3% sucrose
0.8% agar
pH 5.7

TABLE IX

Results of tomato transformation with Agobacterium tumefaciens LBA4404 containing the binary vector, pMChAD

Vector	# cotyledons	# kan-shoots	# plants rooted
pMChAD	425	117	4

EXAMPLE 6

Generation of transgenic canola plants

The chimeric chitinase gene carried on the binary vector pMChAD in Agrobacterium tumefaciens strain LBA4404, was introduced into canola plants (B. napus, var Westar) by infection of cut hypocotyl sections. The features of plasmid pMChAD have been described in Example 5. Agrobacterium infection of cut hypocotyls was carried out according to the following schedule:

DAY 1

Make germination medium -- 30 mM $CaCl_2$, 1.5% agar. Place 500 ml in a 190 mm crystallizing dish, cover with foil, and autoclave for 20 min. Prepare one dish for every 75 seeds to be planted.

Sterilize seeds by stirring in 10% Clorox, 0.1% SDS for 30 min. Rinse thoroughly with sterile water. Seeds may be sterilized in bulk and dried by placing them in a laminar flow hood in an open sterile dish for several hours. Store in a parafilm-sealed sterile dish in a refrigerator and use as needed.

Plant 75 seeds per crystallizing dish, and place in the dark at 25°C for 5 days.

DAY 5

Start overnight cultures of Agrobacterium by inoculating 3 ml of sterile liquid Min A medium with single colonies grown on the appropriate selective medium. Shake at 250 RPM at 28°C. for 18-20 hours.

Make co-cultivation medium BC-1 (Table X) containing 100 µM acetosyringone. Acetosyringone is kept as a 100 mM stock in DMSO for a maximum of three weeks. Filter-sterilize and add after autoclaving.

After plates have solidified, dry them by leaving open in a laminar flow hood for 30 min., and then score 2 cm grooves in agarose into which hypocotyl pieces will be placed. Make 10 grooves per plate.

Make bacterial dilution medium, MS liquid (Gibco), containing 100 µM acetosyringone and filter-sterilize.

DAY 6

For each bacterial strain to be used in the transformation, place 22.5 ml bacterial dilution medium into a sterile dish.

Cut seedling hypocotyls into 1 cm segments and place immediately into bacterial dilution medium.

Add 2.5 ml of Agrobacterium overnight culture (OD_{650} = 1.0 to 2.0) to each plate of bacterial dilution medium containing hypocotyl pieces. Final bacterial concentration is about 10% cells per ml.

After about 30 min., remove hypocotyl pieces from the bacterial suspension and place in grooves on co-cultivation medium. DO NOT blot pieces dry. Dried surface of agarose will absorb excess liquid.

Incubate co-cultivation plates for three days in dim light at 25°C.

DAY 8

Make selective media which is BC-1 containing an appropriate antibiotic for control of Agrobacterium growth and the appropriate plant cell growth inhibitor to select for transformed tissue growth. In this experiment, 500 mg/l cefotaxime was used to inhibit growth of Agrobacteria and kanamycin (100 mg/l) was used to inhibit growth of untransformed cells. Score grooves in agarose as with co-cultivation plates.

Make explant washing solution -- liquid MS containing either 500 mg/l cefotaxime or 500 mg/l carbenicillin. Prepare a volume equal to the number of co-cultivation plates times 20 mls, and filter-sterilize.

DAY 9

Label a set of sterile culture dishes to correspond exactly to the co-cultivation plates. Distribute 20 mls washing medium into each dish.

Transfer the ten explants from each co-cultivation plate to the corresponding dish containing washing medium. Shake slowly for three hours to wash Agrobacterium from explants.

Transfer to selective BC-1 plates and place in 16:8 hr. light:dark chamber at 25°C.

DAY 29

Prepare fresh selective BC-1 media as on Day 8.

DAY 30

Record observations of callus growth from cut ends and transfer explants to fresh media.

DAY 50

Prepare fresh selective BC-1 media.

DAY 51

Record observations of callus growth from cut ends and transfer explants to fresh media.

After three weeks on selective medium, 40/200 cut hypocotyl ends were found to give rise to kanamycin resistant callus tissue. After eight weeks, the total number of kanamycin resistant calli per cut hypocotyl end was 129/200. Shoot induction was initiated on the kanamycin resistant calli when they were at least 5 mm in diameter by transferring to BS-5 shoot induction medium (Table X). These cultures were maintained in continuous light at 25°C during the induction period. The explants were transferred to fresh BS-5 medium every two weeks. A total of 64 explants from B. napus var Westar were placed on shoot induction medium.

Approximately 5-6 weeks after culturing on shoot induction medium, recognizable shoot primordia appeared. These were allowed to elongate somewhat before being excised from the callus tissue. Shoots initially appear highly vitrified -- thick, translucent, glassy leaf and stem tissue. In order to "normalize" the tissue, the shoots were subcultured for at least two three-week cycles on MSV-1A medium (Table X). The shoot tip and several internodes below were transferred during subculture. Normalization occurs most efficiently under short photoperiods ie., 10 hr light/14 hr dark or 12 hr light/ 12 hr dark. This photoperiod also prevents flowering. A total of 20 shoots were placed on "normalization" medium.

Since B. napus forms roots very inefficiently in culture, normalized shoots were planted directly into potting mix without attempting to root in vitro . The shoot was excised near the agar surface, the cut surface dipped in Rootone, and the shoot planted in water-saturated Metro-mix in an 8 inch pot. The pot

was covered with a plastic bag until the plant was clearly growing. Three transgenic B. napus plants were obtained using this procedure and were grown in the greenhouse. These plants were analyzed for expression of the chimeric chitinase gene by extracting soluble leaf protein and assaying for the presence of the bean chitinase polypeptide by reaction with anti-chitinase antibodies. The results of this experiment are presented in Figure 10. Protein extracts of untransformed B. napus did not contain any immunoreactive polypeptides whereas two of three transgenic plants containing the chimeric chitinase gene expressed the bean enzyme constitutively. The size of the immunoreactive protein in the Canola extracts was identical to that of the protein found in ethylene-treated bean plants. This indicates that the signal peptide normally present on the precursor protein was efficiently cleaved in Canola and suggests that the mature protein was localized in the vacuole of the heterologous plant.

TABLE X

CANOLA TISSUE CULTURE MEDIA

BC-1 (Callus Growth Medium)

per liter:

MS Minimal Organic Medium (MS salts, 100 mg/L i-inositol, 0.4 mg/L thiamine)
30 G/L Sucrose
18 G/L Mannitol
0.2 mg/L 1,4-D
3 mg/L Kinetin
0.6% (3 G/500 ml) DNA-Grade Agarose
pH 5.8

BS-5 (Callus Shoot Induction Medium)

per liter:

K3 Macronutrients	100 ml 10X stock
MS Micronutrients	1 ml 1000X stock
6.3 mM $CaCl_2$-$2H_2O$	10 ml 0.63 M stock (46.1 G/500 ml)
100 µM Na_2EDTA	10 ml 10 mM stock (1.86 G/500 ml)
100 µM $FeSO_4$-$7H_2O$	10 ml 10 mM stock (1.39 G/500 ml)
T Vitamins	1 ml 1000X stock

250 mg/L Xylose
10 G/L Sucrose
0.6 G/L MES
0.25% (1.25 G/500 ml) DNA Grade Agarose
pH 5.7

2 mg/L Zeatin	Add after autoclaving
0.1 mg/L IAA	Add after autoclaving

MSV-1A (Shoot Maintenance Medium)

per liter:

MS Minimal Organic Medium (MS Salts, 100 mg/L i-inositol, 0.4 mg/L thiamine)
10 G/L Sucrose
T Vitamins 1 ml 1000X stock
0.4% (2 G/500 ml) DNA-Grade Agarose
pH 5.8

TABLE X (continued)

Brassica napus Stock solutions

Stock	Ingredient	(Stock)	(Final) (In Medium)	Amount/ liter (Stock)
MS Major Salts	NH_4NO_3	10x	20.6 mM	16.5 gm
	KNO_3		18.8	19.0
	$MgSO_4$-$7H_2O$		1.5	3.7
	KH_2PO_4		1.25	1.7
	$CaCl_2$-$2H_2O$		3.0	4.4
K3 Major Salts	KNO_3	10x	25.0 mM	25.0 gm
	$(NH_4)_2SO_4$		1.0	1.34
	$MgSO_4$-$7H_2O$		1.0	2.5
	KH_2PO_4		1.5	2.01
	NH_4NO_3		3.1	2.5
CaCl2-2H2O	$CaCl_2$-$2H_2O$	100x	6.3 mM	92.3 gm
MS Micro-nutrients	MnCl2-4H2O	1000x	100 µM	19800 mg
	H_3BO_3		100	6200
	$ZnSO_4$-$7H_2O$		30	8625
	KI		5	830
	$NaMoO_4$-$2H_2O$		1.2	250
	$CuSO_4$-$5H_2O$		0.1	25
	$CoCl_2$-$6H_2O$		0.1	25
Fe EDTA	Na_2-EDTA	100x	100 µM	3.73 gm
	$FeSO_4$-$7H_2O$		100	2.78
I Vitamins	Myo-Inositol	100x	100 mg/l	10000 mg
	Thiamine		0.5	50
	Glycine		2.0	200
	Nicotinic acid		5.0	500
	Pyrodoxine		0.5	50
	Folic acid		0.5	50
	Biotin		0.05	5

TABLE X (continued)

CANOLA TISSUE CULTURE VITAMIN STOCKS

B5 Vitamins (1000X) 100 ml in H_2O

Nicotinic Acid (Shelf)	100 mg/100 ml
Thiamine Hydrochloride (Shelf)	1000 mg/100 ml
Pyridoxine Hydrochloride (Freezer, dessicator)	100 mg/100 ml
M-Inositol (Shelf)	10,000 mg/100 ml

T+ Vitamins (1000X) 100 ml in H_2O

Biotin (Refrigerator, dessicator) 5 mg/100 ml	
Pyridoxine Hydrochloride (Freezer, dessicator)	50 mg/100 ml
Thiamine Hydrochloride (Shelf)	50 mg/100 ml
Nicotinic Acid (Shelf)	500 mg/100 ml
Folic Acid (Shelf)	50 mg/100 ml
Glycine (Shelf)	200 mg/100 ml
M-Inositol (Shelf)	10,000 mg/100 ml

K3 Vitamins (1000X) 100 ml in H_2O

Pyridoxine Hydrochloride (Freezer, dessicator)	10 mg/100 ml
Thiamine Hydrochloride (Shelf)	100 mg/100 ml
Nicotinic Acid (Shelf)	10 mg/100 ml
M-Inositol (Shelf)	10,000 mg/100 ml

Dispense 5 ml aliquots into scintillation vials; label each vial with color-coded tape.

EXAMPLE 7

Resistance of transgenic canola to Rhizoctonia solani

In this example, transgenic canola plants of Example 6 are shown to be resistant to infection by Rhizoctonia solani. The resistant phenotype of these transgenic plants is characterized by a delay in the appearance of disease and a reduction in disease severity.

Due to limited seed production by the primary transformants and unavailability of homozygous stocks, R1 progeny derived from two independent transformants (#9 and #10) were used to test for fungal resistance. Since the transgenic B. napus plants of Example 6 also contained an ALS gene encoding resistance to the sulfonylurea herbicides, the segregation ratio of the R1 progeny was determined by scoring for herbicide resistance. Seeds were surface sterilized as outlined in the transformation procedure of Example 6. The seed were then placed on MSV-1A medium (TABLE X) containing 10 ppb chlorsulfuron in Magenta boxes. Approximately 30-40 seeds were used and divided between two Magenta boxes. Plants were allowed to germinate and grow for approximately two weeks with a 16 hr. photoperiod at 25°C. Seedlings which displayed elongated hypocotyls (6-12 cm), expanded cotyledons, true leaf formation, and well developed root systems were scored as resistant. Seedlings scored as sensitive displayed short hypocotyls (1-2 cm), small purplish-green cotyledons, no true leaf formation, and severely stunted roots which did not penetrate the surface of the agar culture medium. The results of such an analysis for transformants #9 and #10 are shown in TABLE XI. The segregation ratio of 3 resistant:1

sensitive for transformant #10 indicates the presence of a single insertion site of pMChAD T-DNA in these plants. The ratio of 9.5 resistant:1 sensitive, found for transformant #9, is close to the expected 15:1 ratio for transformants containing two independent insertions of pMChAD T-DNA. Due to limited seed supply both resistant and sensitive seed of transformants #9 and #10 were combined and used for fungal resistance studies; the remaining seed were germinated and allowed to grow to maturity in a greenhouse to provide homozygous lines for future analysis.

To determine the level of resistance of transgenic canola plants containing the bean chitinase gene, modified according to Example 1 for constitutive expression in plants, 16 of the pooled R1 seed were germinated in soil and grown in a growth chamber for 14 days at 20°C with a photoperiod of 16 hr. day: 8 hr. night. The seedlings were transplanted into soil containing 0.75 ml per liter R. solani inoculum (prepared as described in Example 3). This level of inoculum was determined empirically to result in the survival of approximately 50% of the transplanted seedlings when wild type B. napus cv. Westar was used. In contrast to tobacco, canola is extremely sensitive to infection by R. solani and lower levels of inoculum were required in these experiments. The extent of disease was monitored by recording the number of surviving plants at various time intervals following infection. The results of two independent experiments are shown in Figure 11 and indicate that transgenic canola plants containing the modified chitinase gene exhibit increased survival rates in the presence of fungal inoculum

when compared to wild type canola. In the first experiment, 88% of the transgenic plants survived infection while only 67% of the wild type canola plants survived. Analysis of the surviving plants indicated that both wild type and transgenic plants contained lesions caused by Rhizoctonia; however, the severity of the lesions produced on the wild type plants (disease index of 3.8) was greater than those produced on the transgenic plants (disease index of 2.6). A disease index of 0 is indicative of no infection while an index of 5 indicates severe root rot (Gugel, R. K., Yitbarek, P. R. Verma, Morrall, R.A.A. and Sadasivaiah, R. S. (1987) Can. J. Plant Path. 9:119-128). At present,it is not known whether transgenic plants which did not survive fungal infection lacked the modified chitinase gene as a result of genetic segregation. However, the availability of homozygous lines of these and other transgenic canola plants containing the modified chitinase gene of the present invention should result in even higher levels of resistance and allow more quantitative evaluations of the resistant phenotype. Although the limitations of working with segregating populations of plants are recognized, the data presented in this example clearly show that transgenic plants exhibit increased survival rates and a delay in disease development when grown in infested soil. This is likely to be of important practical value by enabling canola seedlings to survive the critical period during stand establishment when they are most susceptible to attack by soil-borne pathogens.

TABLE XI

Segregation data of transgenic Brassica napus lines #9 and #10

	MSC-1A + 10 ppm chlorsulfuron
1. Wild type (cv. Westar)	
Sensitive	10/10
Resistant	0/10
2. pMChAD#9	
Sensitive	4/42
Resistant	38/42
3. pMChAD#10	
Sensitive	9/37
Resistant	28/37

EXAMPLE 8

Transformation of chimeric chitinase gene into rice cells.

Another established means of introducing DNA into plants is by direct DNA uptake into protoplasts. Protoplasts derived from rice suspension cultures were used to introduce the chimeric chitinase gene, carried on the plasmid pK35CHN, into rice. Suspension cultures were initiated from anther-derived callus and maintained by weekly subculture into liquid N6 medium (Table XII) containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% sucrose, pH 6.0. Protoplasts were isolated from suspension cultures 4-5 days after subculturing using a mixture of the cell wall degrading enzymes cellulase and macerozyme. Four mls of the enzyme mixture consisting of 2% (wt/vol) cellulase "Onozuka" RS and 0.5% (wt/vol) Macerozyme (both from Vakult Homsh, Nishinomiya, Japan) in 13% mannitol pH 5.6, were used per gram of cells. The mixture was incubated on a rotary shaker (30 rpm) at 25°C for 16-18 hours. Released protoplasts were filtered through a 60 µm nylon mesh, transferred to 50 ml Pyrex test tubes and washed twice by centrifugation at 80 x g for 10 minutes in Krens F solution (Table XII). Protoplasts were purified by resuspending the pellet in N6 medium containing 2 mg/l 2,4-D and 17% (wt/vol) sucrose, centrifuging at 80 x g for 20 minutes and collecting the floating layer. Cell counts were made with a hemocytometer and indicated a yield of approximately 3.8×10^6 protoplasts per gram of starting tissue.

Purified protoplasts were resuspended at a density of 1×10^6 /ml in Krens F solution and placed in a waterbath at 45°C for 5 min. After cooling in

ice for 10-20 seconds the protoplasts were dispensed into 15 ml polystyrene tubes, in 1 ml aliquots. 25 µg of calf thymus DNA and 10 µg of pK35CHN plasmid were pipetted into each tube, and mixed well before the addition of 0.5 ml 40% (wt/vol) polyethylene glycol (MW 8000). After incubation at room temperature, the protoplasts were slowly diluted and then washed twice with Krens F solution.

Protoplasts were resuspended at 10^6/ml in N6 medium containing 17% sucrose and 2mg/liter 2,4-D, pH 5.8. An equal volume of molten 2.5% (wt/vol) Seaplaque agarose in the same medium was mixed with the protoplasts to give a plating density of 5 x 10^5 /ml. Aliquots of this mixture were plated in 6 cm diameter petri dishes and were allowed to solidify. The agarose blocks were cut into 1 x 1 cm segments which were placed in the 3.5 cm diameter wells of a 3 x 2 well cluster dish (Gibco). Protoplast division was supported by immersing the slabs in culture medium containing 0.1 g of undigested suspension cells and incubating in the cluster dishes on a rotary shaker at 30 rpm, in darkness at 25°C. The agarose slabs were transferred to fresh culture medium without nurse cells after two weeks. Clusters of 20 or more cells were visible after 3 weeks, at which stage 100 µg/ml kanamycin sulfate was included in the culture medium. By 8 weeks, the agarose slabs were transferred onto the surface of agarose-solified N6 medium containing 2mg/liter 2,4-D and 8% (wt/vol) sucrose with 100ug/ml kanamycin sulfate. After 10 weeks, the most vigorous colonies were individually transferred to fresh agarose-solidified medium. 92 individual kanamycin tolerant calli were recovered from 5 x 10^6 protoplasts.

Protein was extracted from five different samples of kanamycin resistant calli as described in Example 2 and assayed for expression of the bean chitinase polypeptide by immunological methods. The results, presented in Figure 12, indicate the presence of an immunoreactive polypeptide, somewhat larger than the bean chitinase polypeptide, in untransformed rice cells. This may represent an endogenous rice chitinase enzyme. Western blot analysis of proteins extracted from rice leaves indicate that this protein is not expressed in uninfected leaf tissue. In addition to this polypeptide, rice cells transformed with pK35CHN also contain an additional immunoreactive polypeptide which co-migrates with purified bean chitinase. This indicates that the precursor form, encoded by the chimeric chitinase gene is processed correctly in monocot cells and suggests that it is localized in the plant vacuole. The lower molecular weight bands present in this experiment are likely due to proteolytic breakdown of the bean chitinase enzyme during protein isolation.

The cell cultures used as a source of protoplasts for this transformation experiment lost their ability to regenerate into rice plants. However, Ruslan Abdulla, Edward C. Cocking and John A. Thompson (Biotechnology (1986) 4:1087) and Junko Kyozuko, Yasuyki Hayashi and Ko Shimamoto (Mol. Gen. Genet. (1987) 206:408) disclose methods for regenerating rice plants from protoplasts. Efficient and reproducible plant regeneration can be achieved from protoplast-derived colonies after transfer to a hormone-free medium. The process of plant regeneration occurs through somatic embryogenesis of the protoplast-derived calli. Therefore, one skilled

in the art could obtain rice plants containing the recombinant DNA construct of the present invention through protoplast transformation using cell cultures capable of regeneration.

TABLE XII

Rice Tissue Culture Medium

Kren's F Solution

(a) Use 1 Litre Bottle

NaCl	8.12 g
KCl	0.27
$Na_2HPO_4.7H_2O$	0.20
Glucose	0.90

Make up to 500 ml H_2O
(pH adjusted to 5.8)
Autoclave

(b) Use 500 ml Bottle

$CaCl_2.2H_2O$	18.36 g

Make up to 500 ml H_2O
Autoclave

When cool, add (b) to (a)
Make up to 1 liter H_2O
Label KREN'S F

PEG Solution in Kren's F

Use 100 ml Bottle

Polyethylene Glycol (M.Wt 8000)	40.00 g

Make up to 100 ml KREN'S F
Autoclave

N6 Medium

Salts	mg/l	mM
Major elements		
$(NH_4)_2SO_4$	463	3.5
KNO_3	2830	28.0
$CaCl_2 \cdot 2H_2O$	166	1.13
$MgSO_4 \cdot 7H_2O$	185	0.75
KH_2PO_4	400	2.94
$NA_2 \cdot EDTA$	37.3	0.20(Na)
$FeSO_4 \cdot 7H_2O$	17.8	0.10(Fe)

TABLE XII (continued)

Minor elements		
H_3BO_3	1.6	25.8
$MnSO_4 \cdot 1H_2O$	3.3	19.5
$ZnSO_4 \cdot 7H_2O$	1.5	5.2
KI	0.8	5.0
Organic constituents		
Thiamine hydrochloride	1.0	
Glycine	2.0	
Pyridoxine	0.5	
Nicotinic acid	0.5	
2,4-D	2.0	
Sucrose	30 g/l	

pH: Adjusted to 5.8 with NaOH (if too acid) or HCl (if too basic).

From Chu et al. (1975) as modified by Armstrong and Green (1984).

Cell Suspension medium	30 g/l sucrose
Protoplast Floatation and culture medium	170 g/l sucrose
Callus culture medium	80 g/l sucrose and 0.4% seaplaque low melting point agarose

CLAIMS

What is claimed is:

1. A recombinant DNA construct capable of transforming a plant comprising the following DNA fragments: (a) a high level promoter operably linked to (b) a plant chitinase gene wherein said high level promoter causes the overexpression of plant chitinase polypeptide thereby conferring resistance to plant pathogenic fungi.

2. A recombinant DNA construct of Claim 1 wherein the high level promoter is derived from the genome of a virus.

3. A recombinant DNA construct of Claim 1 wherein the high level promoter is derived from the opine synthase genes of Agrobacterium.

4. A recombinant DNA construct of Claim 1 wherein said high level promoter is selected from the group consisting of the 35S and 19S constituents of the cauliflower mosaic virus, the NOS and OCS promoters of the opine synthase genes of Agrobacterium, the promoter of the RUBISCO small subunit, and the promoter from the chlorophyll A/B binding protein genes.

5. A recombinant DNA construct of Claim 1 wherein the high level promoter also contains an enhancer to further increase transcription and expression.

6. A recombinant DNA construct of Claim 5 wherein the enhancer is derived from the genome of a virus.

7. A recombinant DNA construct of Claim 6 wherein the enhancer is derived from the 35S promoter of the cauliflower mosaic virus.

8. A recombinant DNA construct of Claim 5 wherein the enhancer is derived from the opine synthase genes of Agrobacterium.

9. A recombinant DNA construct of Claim 1 wherein the high level promoter is a tissue specific promoter.

10. A recombinant DNA construct of Claim 9 wherein the tissue specific promoter is root specific.

11. A recombinant DNA construct of Claim 9 wherein the tissue specific promoter is leaf specific.

12. A recombinant DNA construct of Claim 9 wherein the tissue specific promoter is stem specific.

13. A recombinant DNA construct of Claim 9 wherein the tissue specific promoter is seed specific.

14. A recombinant DNA construct of Claim 9 wherein the tissue specific promoter is petal specific.

15. The recombinant DNA construct of Claim 1 wherein said high level promoter is the 35S constituent of the cauliflower mosaic virus, and said plant chitinase gene is derived from a bean plant.

16. A recombinant DNA construct of Claim 1 comprising a plasmid selected from the group consisting of pK35CHN641 and pK35CHN695.

17. A transgenic plant containing a recombinant DNA construct of Claims 1-15 or 16.

18. A transgenic plant containing a recombinant DNA construct of Claim 1 wherein said plant is a monocotyledonous plant selected from the group consisting of corn, alfalfa, oats, millet, wheat, rice, barley, and sorghum.

19. A transgenic plant containing a recombinant DNA construct of Claim 1 wherein said plant is a dicotyledonous plant selected from the group consisting of soybean, tobacco, petunia,

cotton, sugarbeet, sunflower, carrot, celery, flax, cabbage, cucumber, pepper, tomato, potato, brassica, bean, strawberry, and lettuce.

20. A transgenic tobacco plant containing the recombinant DNA construct of Claim 15.

21. A transgenic rice plant containing the recombinant DNA construct of Claim 15.

22. A transgenic canola plant containing the recombinant DNA construct of Claim 15.

23. A transgenic tomato plant containing the recombinant DNA construct of Claim 15.

24. Seed obtained by growing a transgenic plant of Claims 17-22 or 23.

25. A recombinant DNA construct of Claim 1, wherein said plant pathogenic fungi is a soil pathogen.

26. A transgenic plant of Claims 20-22 or 23 wherein said plant is resistant to soil pathogenic fungi.

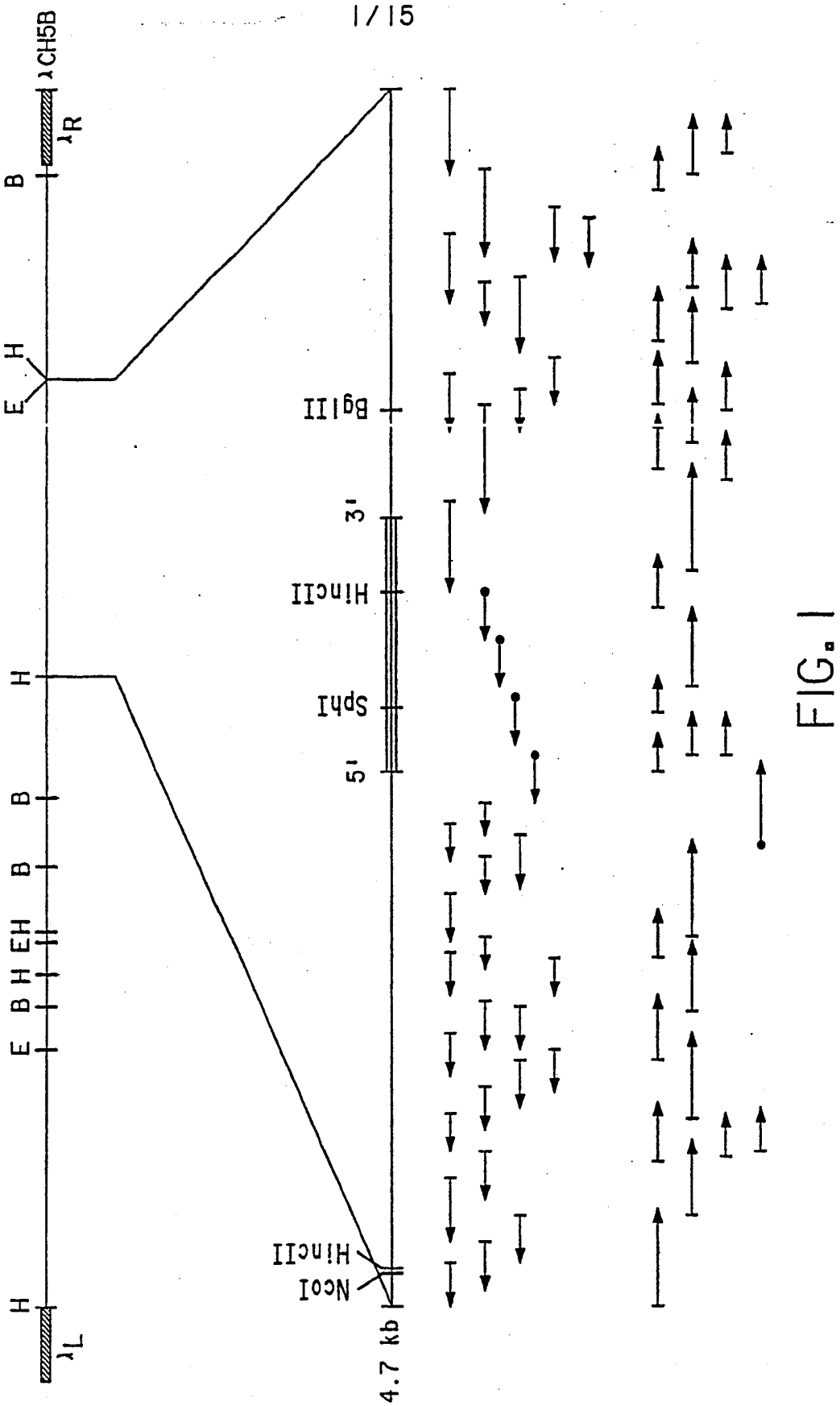
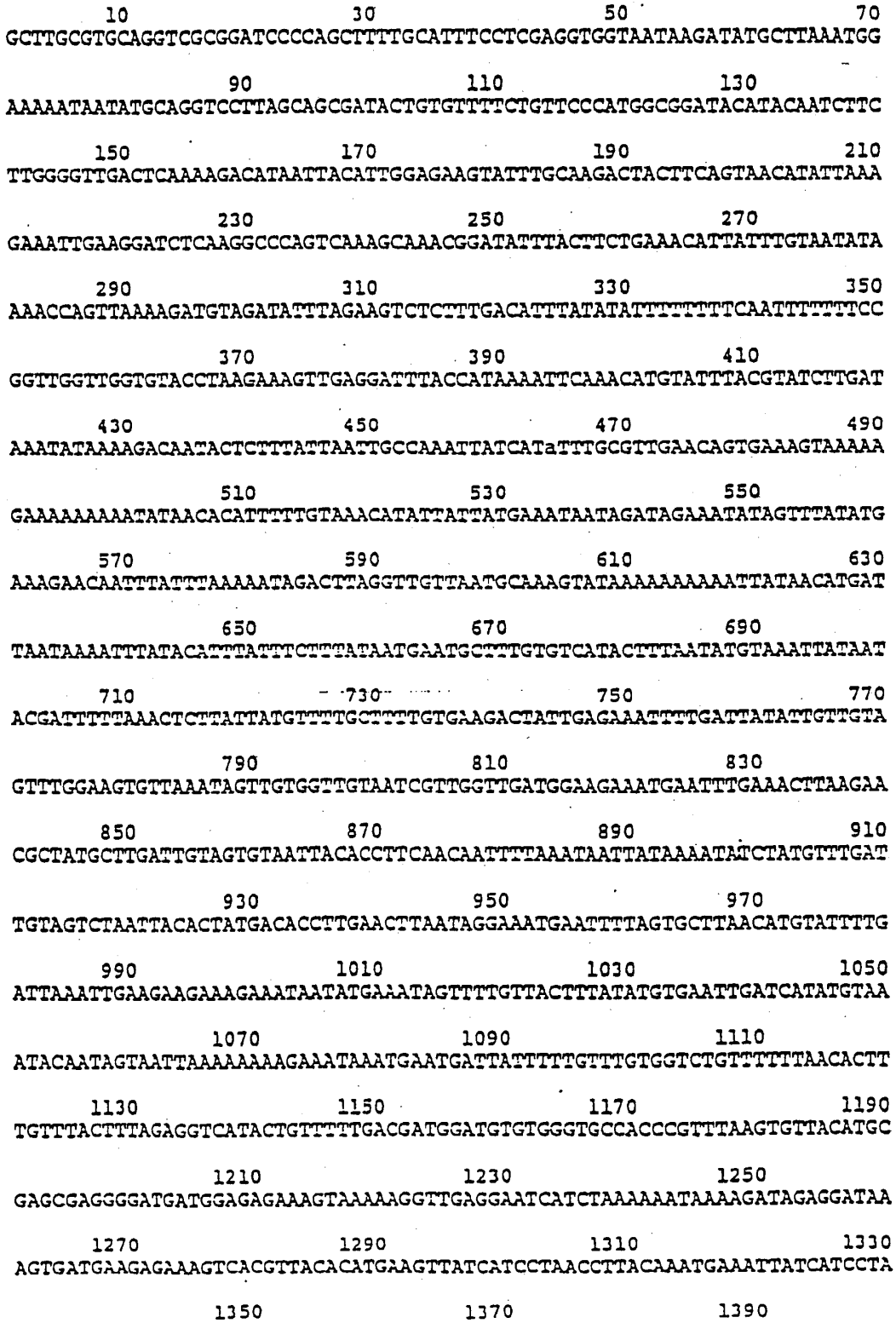

λCH5B
λR
B
H
E
BglII
3'
HincII
SphI
5'
H
B
B
EH
H
E B H
H
NcoI
HincII
4.7 kb
λL

FIG. 1

FIG. 2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30
50
70
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90
110
130
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150
170
190
210
TTGGGGTTGACTCAAAAGACATAATTACATTGGAGAAGTATTTGCAAGACTACTTCAGTAACATATTAAA
230
250
270
GAAATTGAAGGATCTCAAGGCCCAGTCAAAGCAAACGGATATTTACTTCTGAAACATTATTTGTAATATA
290
310
330
350
AAACCAGTTAAAAGATGTAGATATTTAGAAGTCTCTTTGACATTTATATATTTTTTTTCAATTTTTTTCC
370
390
410
GGTTGGTTGGTGTACCTAAGAAAGTTGAGGATTTACCATAAAATTCAAACATGTATTTACGTATCTTGAT
430
450
470
490
AAATATAAAAGACAATACTCTTTATTAATTGCCAAATTATCATaTTTGCGTTGAACAGTGAAAGTAAAAA
510
530
550
GAAAAAAAAATATAACACATTTTTGTAAACATATTATTATGAAATAATAGATAGAAATATAGTTTATATG
570
590
610
630
AAAGAACAATTTATTTAAAAATAGACTTAGGTTGTTAATGCAAAGTATAAAAAAAAAATTATAACATGAT
650
670
690
TAATAAAATTTATACATTTATTTCTTTATAATGAATGCTTTGTGTCATACTTTAATATGTAAATTATAAT
710
730
750
770
ACGATTTTTAAACTCTTATTATGTTTTGCTTTTGTGAAGACTATTGAGAAATTTTGATTATATTGTTGTA
790
810
830
GTTTGGAAGTGTTAAATAGTTGTGGTTGTAATCGTTGGTTGATGGAAGAAATGAATTTGAAACTTAAGAA
850
870
890
910
CGCTATGCTTGATTGTAGTGTAATTACACCTTCAACAATTTTAAATAATTATAAAATATCTATGTTTGAT
930
950
970
TGTAGTCTAATTACACTATGACACCTTGAACTTAATAGGAAATGAATTTTAGTGCTTAACATGTATTTTG
990
1010
1030
1050
ATTAAATTGAAGAAGAAAGAAATAATATGAAATAGTTTTGTTACTTTATATGTGAATTGATCATATGTAA
1070
1090
1110
ATACAATAGTAATTAAAAAAAAGAAATAAATGAATGATTATTTTTGTTTGTGGTCTGTTTTTTAACACTT
1130
1150
1170
1190
TGTTTACTTTAGAGGTCATACTGTTTTTGACGATGGATGTGTGGGTGCCACCCGTTTAAGTGTTACATGC
1210
1230
1250
GAGCGAGGGGATGATGGAGAGAAAGTAAAAAGGTTGAGGAATCATCTAAAAAATAAAAGATAGAGGATAA
1270
1290
1310
1330
AGTGATGAAGAGAAAGTCACGTTACACATGAAGTTATCATCCTAACCTTACAAATGAAATTATCATCCTA
1350
1370
1390

FIG. 2 (continued)

```
ATCTTCATTTGATTCTTCGAGGCTTCATTTTAAAACTATCTAAATAAATTAACTTATGTAATAAAATTAA

      1410                1430                1450                1470
TTTAAATTAATAAAATAGTAAATAAAAAAAATTATACTTAAAATTAAAATTAAATTTAATTATTTCTTTG

                  1490                1510                1530
AATTTTACATTATTATTTTTTATTTATAAATAAAACTTTAAATTATTTTAATTAACTAAAATATAAACAA

      1550                1570                1590                1610
AATTAATAATTATTTATCCATTTTTTTATATAAAAAAATAAAATTTATAATTTTACTATTTACATTATAA

                  1630                1650                1670
AAAATAATTTAAAATACTCTTACAACTTTCACATCATCCACATATTTTAATAAACTAACTTTCTTCTCTA

      1690                1710                1730                1750
ATTTATATCAATCTACACAACTACAACTTAATTTCGCTCCCCAACCCTTGAATTTTCCACTCCCAAATTC

                  1770                1790                1810
AAAATGATTATCGCTTGGTTCTGGTTCACTGTGTTTGGTCGTGGTCTTCCAGTCTTCACGCTTGGGAAGC

      1830                1850                1870                1890
CGCCGGGGTGGGCCCGCAGAAAGGTTTGGCAATGTTGGGACGGAAGCGGTGTTTTAATAAAATAAAAACA

                  1910                1930                1950
GTTTAGATCAAAACTTGGGAAATGAATAATGAGGGTGAGGTGGAAGGAGGAGAAAAGTAAAATTAAGAAT

      1970                1990                2010                2030
AGTTAATGTTGAAGAAATTGATTATGATGGTAAAGTAAGAGTATAAAAGAGAGAGGGAAGGAAGGGAAAC

                  2050                2070                2090
GGAAAGAGAGAAAGAGAAAGAGAAATGAAGAAGAATAGGATGATGATTATGATATGCAGTGTAGGAGTGG
                                    M   K   K   N   R   M   M   I   M   I   C   S   V   G   V   V

      2110                2130                2150                2170
TGTGGATGCTGTTAGTTGGAGGAAGCTACGGAGAGCAGTGTGGAAGGCAAGCAGGAGGTGCACTCTGCCC
  W   M   L   L   V   G   G   S   Y   G   E   Q   C   G   R   Q   A   G   G   A   L   C   P

                  2190                2210                2230
AGGGGGCAACTGTTGCAGCCAGTTCGGGTGGTGCGGCTCCACCACTGACTACTGCGGCAAGGATTGCCAG
  G   G   N   C   C   S   Q   F   G   W   C   G   S   T   T   D   Y   C   G   K   D   C   Q

      2250                2270                2290                2310
AGCCAGTGCGGGGGACCGTCTCCTGCTCCTACTGATCTCAGCGCCCTCATATCCAGGTCCACCTTCGACC
S   Q   C   G   G   P   S   P   A   P   T   D   L   S   A   L   I   S   R   S   T   F   D   Q

                  2330                2350                2370
AGGTGCTCAAACATCGCAACGACGGAGCATGCCCAGCCAAAGGCTTCTACACCTACGATGCCTTCATCGC
  V   L   K   H   R   N   D   G   A   C   P   A   K   G   F   Y   T   Y   D   A   F   I   A

      2390                2410                2430                2450
CGCCGCCAAGGCTTACCCCAGCTTCGGAAACACCGGAGACACGGCCACTCGCAAGAGGGAGATTGCGGCC
  A   A   K   A   Y   P   S   F   G   N   T   G   D   T   A   T   R   K   R   E   I   A   A

                  2470                2490                2510
TTCTTGGGGCAAACGTCTCACGAAACAACCGGGGGATGGGCCACTGCGCCCGACGGACCATACGCATGGG
F   L   G   Q   T   S   H   E   T   T   G   G   W   A   T   A   P   D   G   P   Y   A   W   G

      2530                2550                2570                2590
GATACTGCTTCGTGAGGGAGCGGAACCCCAGTGCGTACTGCTCCGCCACTCCCCAGTTCCCCTGCGCCCC
  Y   C   F   V   R   E   R   N   P   S   A   Y   C   S   A   T   P   Q   F   P   C   A   P
```

FIG. 2 (continued)

```
                2610                2630                2650
TGGGCAGCAGTACTACGGCAGGGGTCCCATCCAGATATCCTGGAACTACAACTATGGTCAGTGCGGAAGG
 G  Q  Q  Y  Y  G  R  G  P  I  Q  I  S  W  N  Y  N  Y  G  Q  C  G  R

     2670                2690                2710                2730
GCCATTGGGGTTGACTTGCTCAACAAACCTGATCTAGTCGCCACTGACTCTGTCATCTCCTTCAAGTCCG
A  I  G  V  D  L  L  N  K  P  D  L  V  A  T  D  S  V  I  S  F  K  S  A

                2750                2770                2790
CCCTCTGGTTCTGGATGACCGCACAGTCCCCCAAACCTTCCTCCCCACGACGTCATCACCTCTCGATGGAC
  L  W  F  W  M  T  A  Q  S  P  K  P  S  S  H  D  V  I  T  S  R  W  T

     2810                2830                2850                2870
CCCCTCCTCTGCCGAGGTCGCCGCCCCCGCCGGGTTCCCCGGCTACGGCACTGTCACCAACATCATCAACGGC
 P  S  S  A  D  V  A  A  R  R  L  P  G  Y  G  T  V  T  N  I  I  N  G

                2890                2910                2930
GGCCTGGAGTGCGGGCGAGGACAGGACAGCAGGGTTCAAGACCGCATCGGATTCTTCAAGAGATACTGTG
G  L  E  C  G  R  G  Q  D  S  R  V  Q  D  R  I  G  F  F  K  R  Y  C  D

     2950                2970                2990                3010
ATCTGCTTGGAGTTGGTTATGGCAACAACCTTGACTGCTACTCTCAGACTCCATTTGGAAATTCACTCTT
  L  L  G  V  G  Y  G  N  N  L  D  C  Y  S  Q  T  P  F  G  N  S  L  F

                3030                3050                3070
CCTCTCTGACCTTGTCACCTCTCAGTGACACTGCCATCCCATCAGAATAAATAAACTCATAAATCTGTGT
 L  S  D  L  V  T  S  Q

     3090                3110                3130                3150
TTCCCTTTCTGATCACAACTTTCCAATAACACTTTTCCCACCATCTATCAATAAATTCACTACTTCTATA

                3170                3190                3210
TACCATATGCACATCAACATGCTACTCTTTTATTTTTATTATTTTTTCATTCATCTGCATACAAATATT

     3230                3250                3270                3290
TTAGTTATTATATAATAAAATGATGAGCTAACTCATTTTCATAAAATAGATTTATGGAAAATTACGTGGG

                3310                3330                3350
ATTTTTAAAAATATTTTTATCTAATAACGGTCCGATAATAGGTGATACGATAGATTCAACAAACATTTAT

     3370                3390                3410                3430
TAGAATCAACTTTTAAACGACTTTAATATATATTAAGAAGCGGACTTTAAACTTACCCATAAAATCAACT

                3450                3470                3490
TATGAATTAAACGACTTTGATATATGTTAAGAAGCGGACTCTAAACTTATCTGACTCATAAAATCAACTT

     3510                3530                3550                3570
ATGAAATGAGATTTACACTCACTTATATAAAATAAAATATCATATATAAGATCTCCAAATATGTCATTTC

                3590                3610                3630
ATTTTCAATTATAAAAATTTAAGTAGATTAAGTCAAATTAATTATATCATATTAATAGTTTTACAATGTA

     3650                3670                3690                3710
TTAATTCAAATTTATTGTATGGTATCGAAGGAGACTCTGTCCATGAAAACATGACAGCATGGATCAAATA

                3730                3750                3770
TCGTAATGAGCACGACAAGGCGAGGGAAGATTCAACCTGCACTGGTATATGTTTCTATCAACTGCAAGAC

     3790                3810                3830                3850
```

FIG. 2 (continued)

```
TTCCAAGACCCGTATCTTTCCCAACCAAAGGAATCTCCATGATTTTTGTGCTACAAATTTTCATAACTTT

                3870                3890                3910
GATGACAGATTCCAAAGTATGGATACACGTTTCCAGAACCTTGATGAACAAATTTAAGTTGTTCAAAACC

      3930                3950                3970                3990
AACTATTTGAGCTGCAGTACAGAAAGGAAGATTGAAGCAAAGTTTTAGTTATCTTTATGCTTATCTTGCT

                4010                4030                4050
TTAATTTTTGGACATCACTTTCCTAATTTTGTTTTTATCTCTTCTTAATTCTATTTGTGAAGAAAAATAG

      4070                4090                4110                4130
GGGAAGAATATGGTTTATGTGTTGTCTTAAAACTGCTATAACTCTGATTGAAGTGTGTTTTAGTTTCTGT

                4150                4170                4190
TGCATATGCTGGATTGATTTTGGTGCTGCAGTTTTGGTAGTTTTGGCATTGTTTGTGGCTGGTTTTTCTA

      4210                4230                4250                4270
CTCTTGTGGTTATGCAGAAAACTAGTTAGTGTGCCATCATAACCTGCTATTAAAGATGCTTTGGGTTTGC

                4290                4310                4330
ATAGTTTCTGTTTTACAGGTGTTGTTTCAAATTCAAACAAGACTAACACAAGCAACCAGGGATTTGTCTT

      4350                4370                4390                4410
CATCAAATAGGGAGAGATTATTTATCAAGAGTGATCAAGAGCTTTGAAGAATCCAAATCTAAGGTGTTTG

                4430                4450                4470
ATGAAAGGATGGAGTGGTTGTTGTGTGTGGGTGGGATTTGGGTAAATTAAAGTGTAATCCACTCCATTGT

      4490                4510                4530                4550
TGAATCTAAAACTGATGTGAATTAAAACCTTTTTGAAAATGGATGTTTTTCTAACCAAGTGAAATAAAAC

                4570                4590                4610
CTGTTGTTTTGTCGAATCAACCAGTTGTTTTACTCTTAGAGGCTTTTGAAAAGGGTTGAAAGCTGTTTGA

      4630                4650                4670                4690
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ATCCTAACAGAATT
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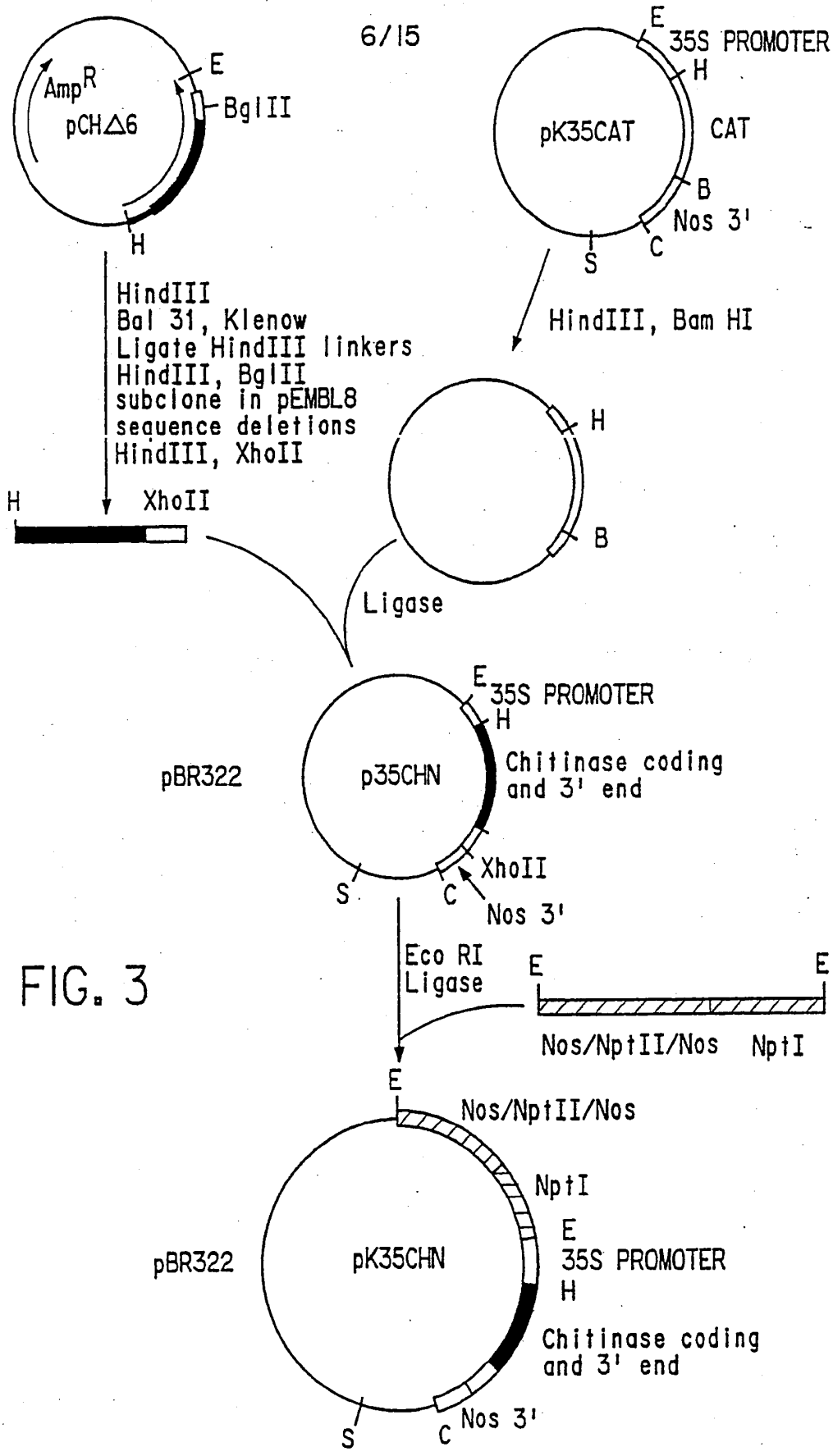

Amp^R
pCHΔ6
E
BglII
H
HindIII
Bal 31, Klenow
Ligate HindIII linkers
HindIII, BglII
subclone in pEMBL8
sequence deletions
HindIII, XhoII
H
XhoII
E
35S PROMOTER
H
pK35CAT
CAT
B
Nos 3'
S
C
HindIII, Bam HI
H
B
Ligase
E
35S PROMOTER
H
pBR322
p35CHN
Chitinase coding
and 3' end
S
C
XhoII
Nos 3'
Eco RI
Ligase
E
E
Nos/NptII/Nos
NptI
E
Nos/NptII/Nos
NptI
E
35S PROMOTER
H
pBR322
pK35CHN
Chitinase coding
and 3' end
S
C
Nos 3'

FIG. 3

FIG. 4

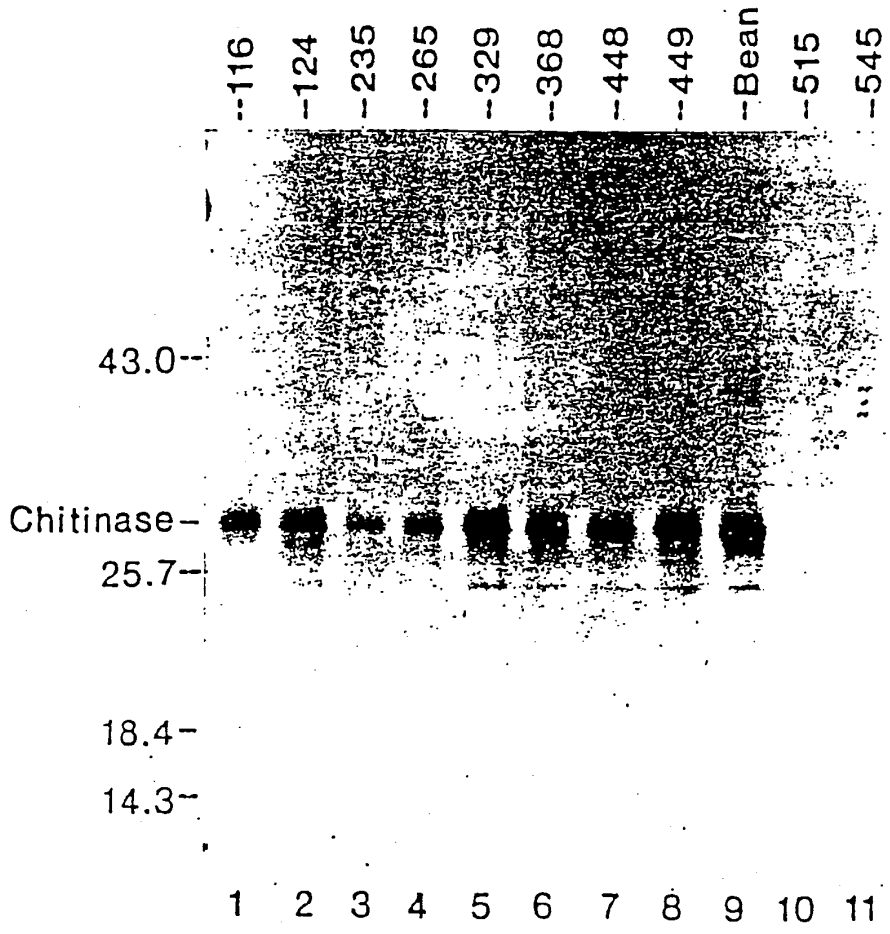


FIG. 5A

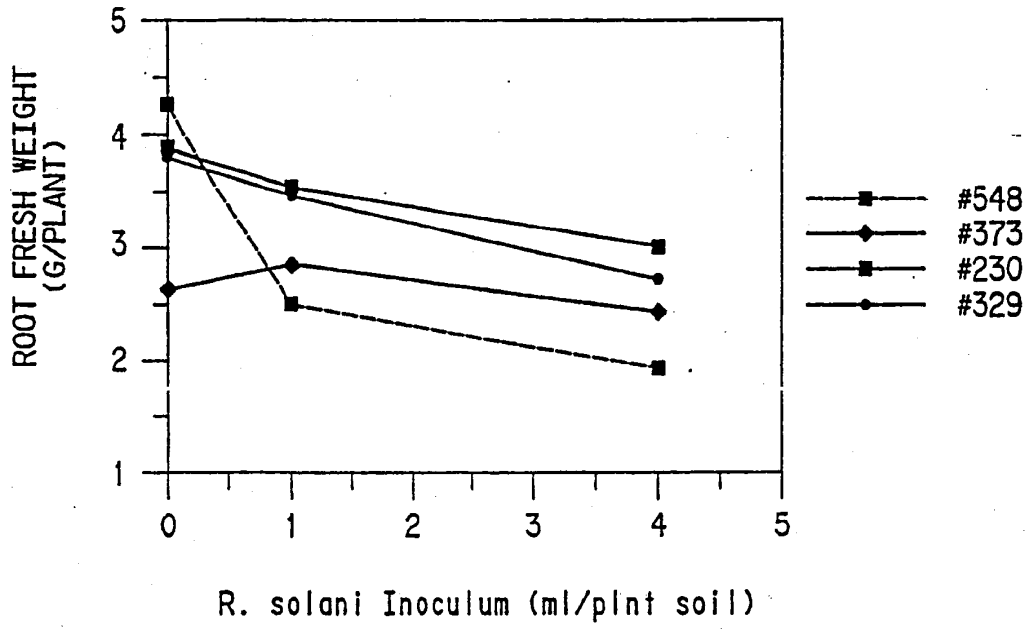

ROOT FRESH WEIGHT (G/PLANT)
5
4
3
2
1
0
1
2
3
4
5
R. solani Inoculum (ml/pint soil)
#548
#373
#230
#329

FIG. 5B

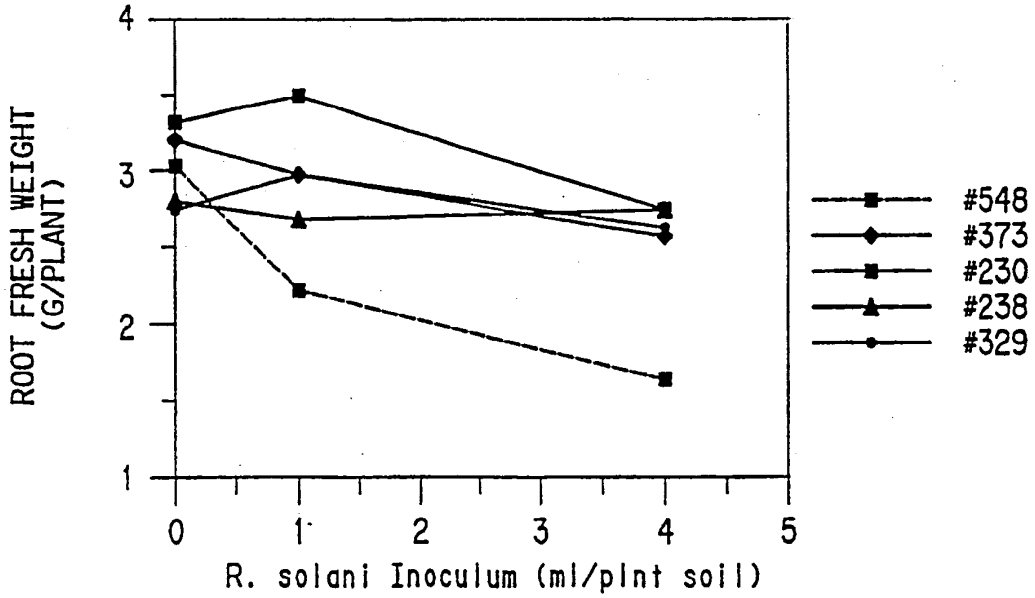
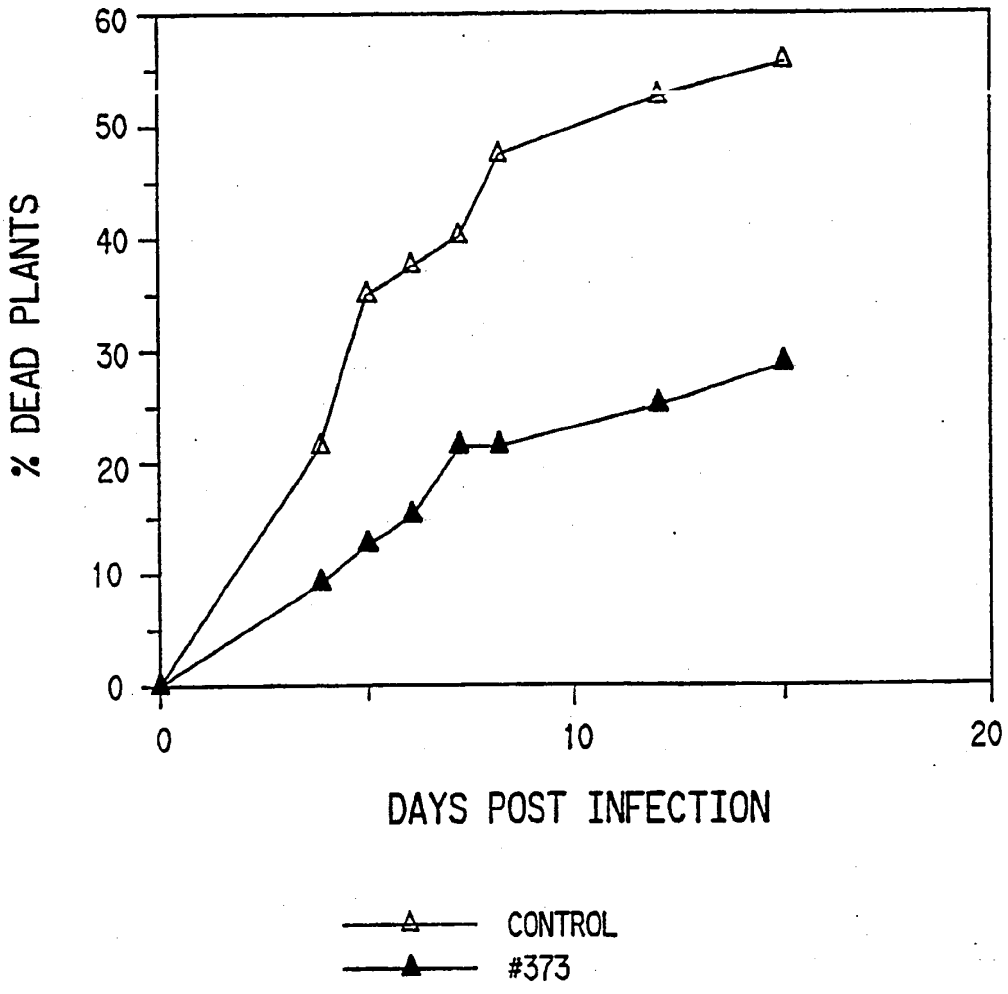

ROOT FRESH WEIGHT (G/PLANT)
4
3
2
1
0
1
2
3
4
5
R. solani Inoculum (ml/pint soil)
#548
#373
#230
#238
#329

FIG. 6


60
50
40
30
20
10
0
% DEAD PLANTS
0
10
20
DAYS POST INFECTION
CONTROL
#373

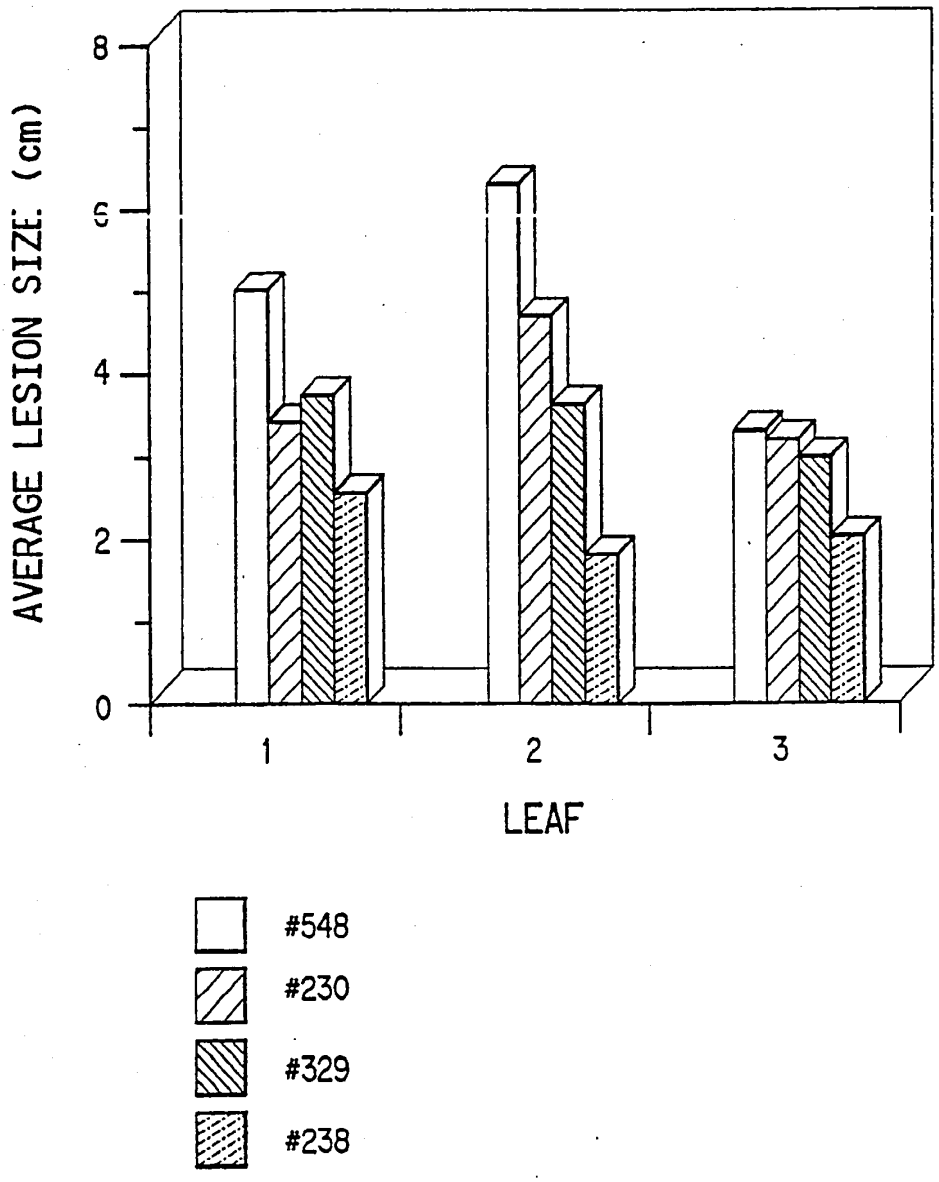


FIG. 7

pMChAD 11/15

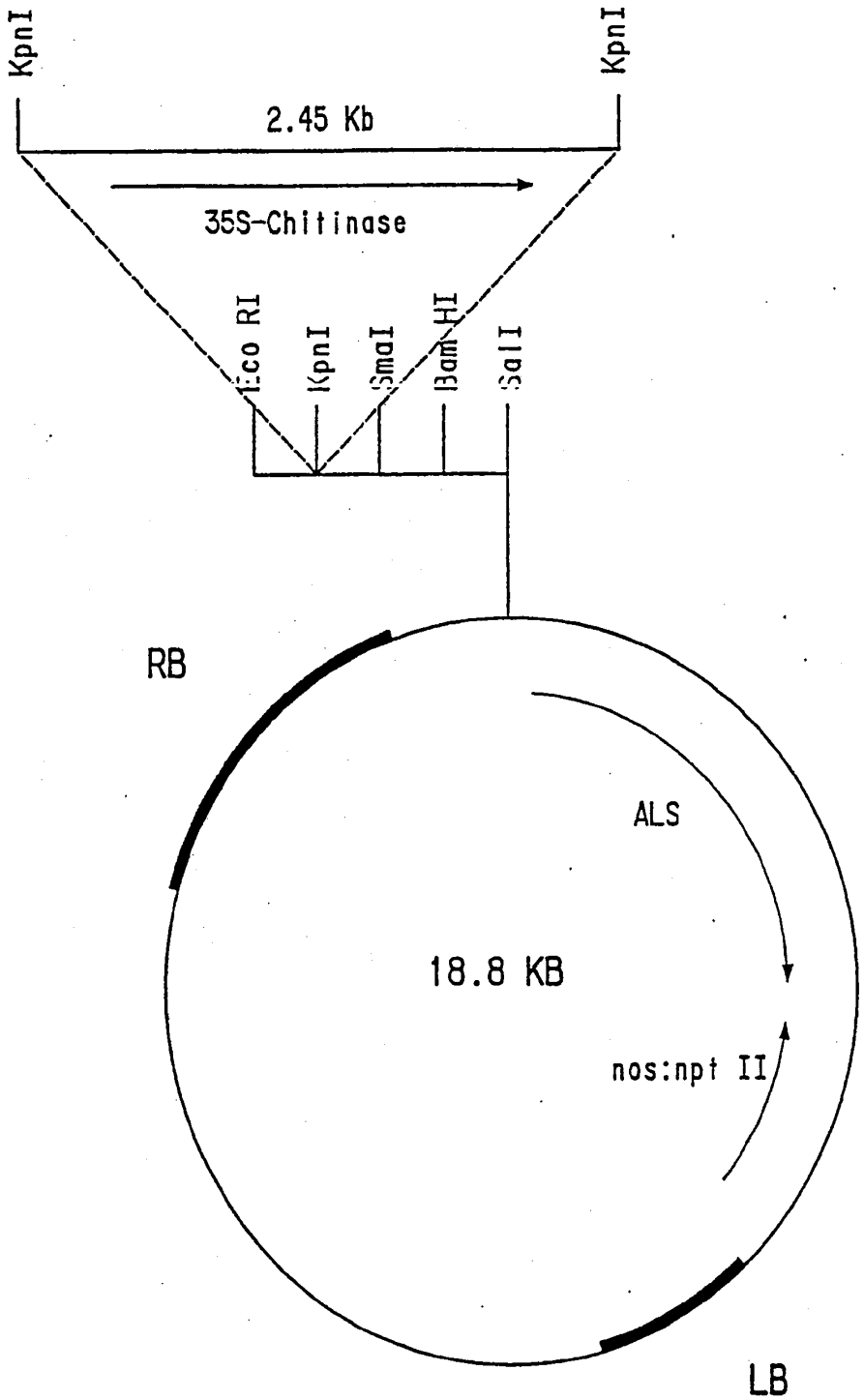


FIG. 8

FIG. 9

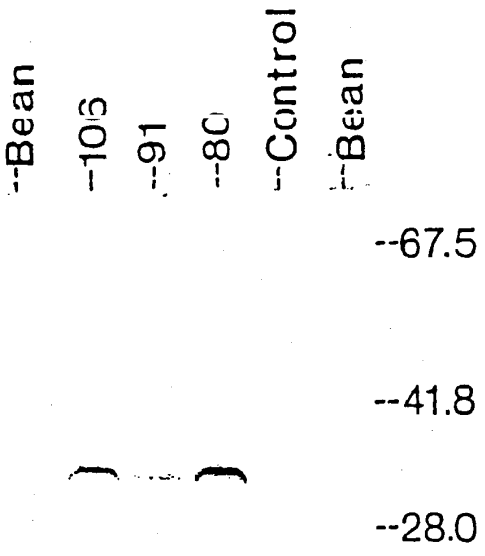


FIG. 10

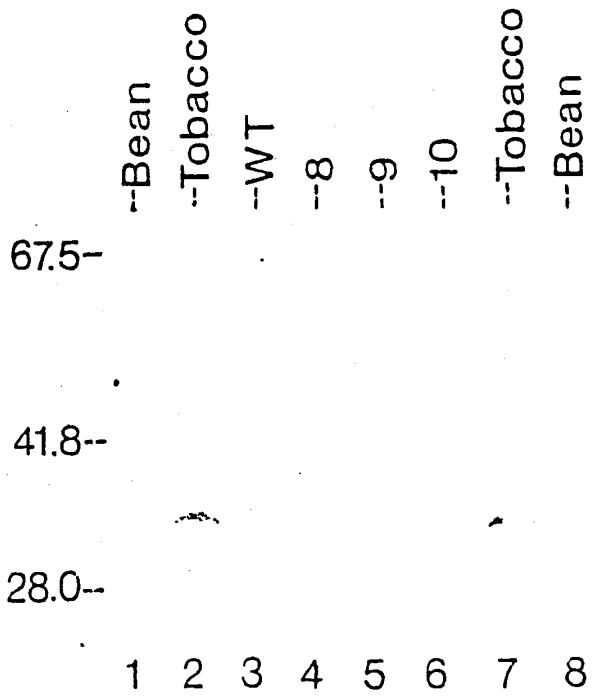


FIG. IIA

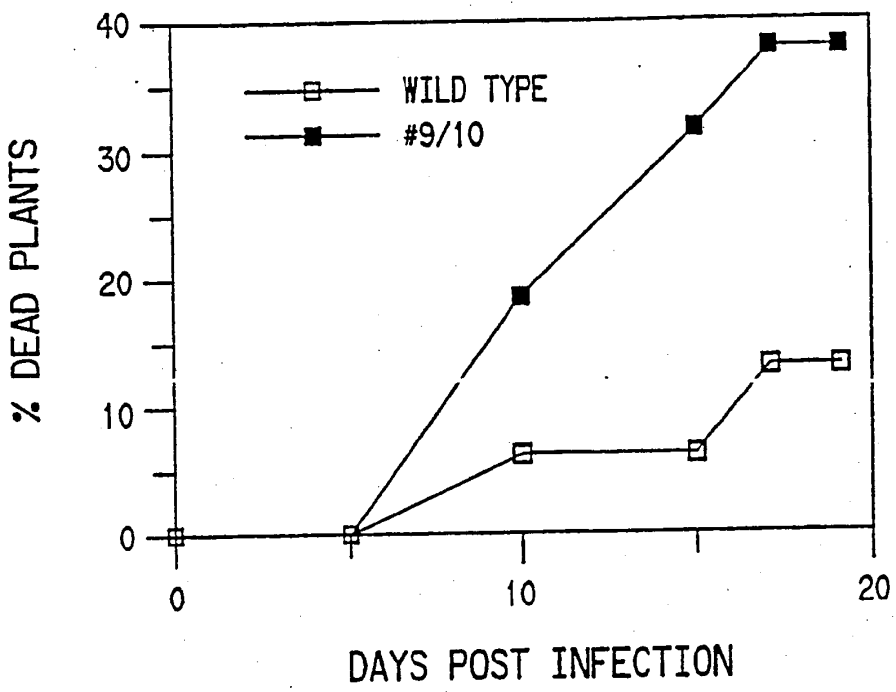


FIG. IIB

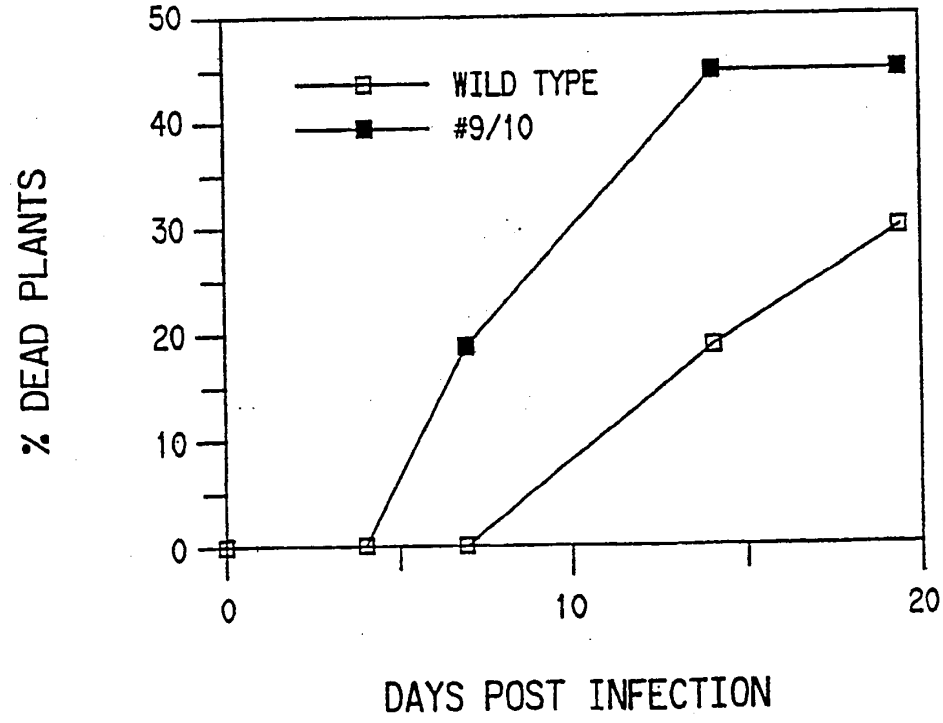
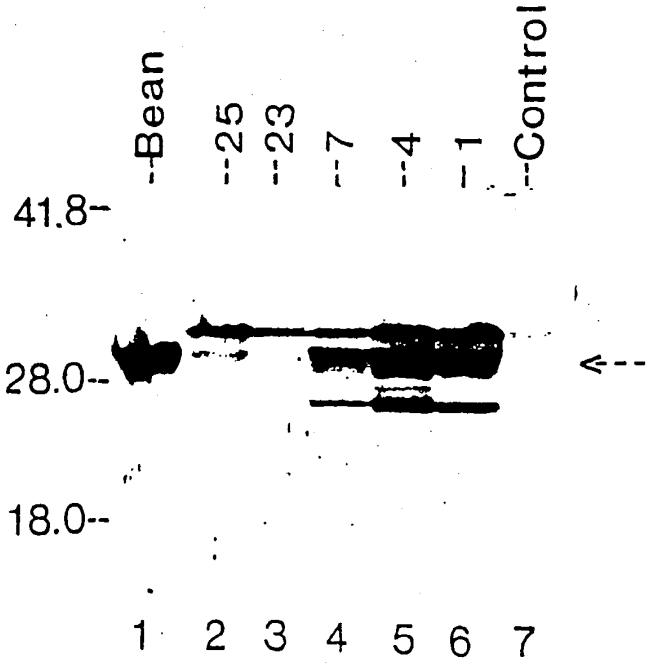


FIG. 12



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/05501

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/56, C 12 N 15/82, A 01 H 5/00, // A 01 N 65/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, A 01 H, A 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0292435 (CIBA-GEIGY) 23 November 1988 see page 14, line 64 - page 15, line 4; page 36, line 58 - page 37, line 22	1,2,4,17, 18
Y		3-15,19-24
Y	Mol. Gen. Genet., volume 210, 1987, Springer-Verlag, J.L. Taylor et al.: "Optimizing the expression of chimeric genes in plant cells", pages 572-577 see "Summary"	3,4,19-23
Y	Mol. Gen. Genet., volume 212, 1988, Springer-Verlag, J.D.G. Jones et al.: "Expression of bacterial chitinase protein in tobacco leaves using two photo-synthetic gene promoters", pages 536-542	4

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th April 1990	- 8 JUN 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS D. SLOWALCZYK

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International Application No. PCT/US 89/05501

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	expression of bacterial chitinase on tobacco susceptibility to leaf brown spot alternaria-longipes", see the abstract, & Phytopathology 78 (12 part 1), 1988, 1556	
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A	Biological Abstracts/RRM, abstract BR29:94361, R. Broglie: "Chitinase could protect against fungal pests", see the abstract, & Genet Technol News 5 (8), 1985, 2	1-26
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A	Chemical Abstracts, volume 109, 1988, (Columbus, Ohio, US), M.H. Harpster et al.: "Relative strengths of the 35 S cauliflower mosaic virus, 1', 2', and nopaline synthase promoters in transformed tobacco, sugarbeet, and oilseed rape callus tissue", see page 152, abstract 32903g, & MGG, Mol. Gen. Genet. 1988, 212(1), 182-90	1,4
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A	EP, A, 0270248 (ICI) 8 June 1988 see page 3, lines 4-12; claims 10,16-19	1-26

International Application No. PCT/US 89/05501

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	see "Summary"; page 536, right-hand column, paragraph 4	
Y	EP, A, 0289479 (MONSANTO) 2 November 1988 see page 16, lines 33-40	5-7
Y	EP, A, 0278659 (LUBRIZOL) 17 August 1988 see the whole document	8
Y	EP, A, 0157351 (AGS) 9 October 1985 see page 16, lines 7-17	9,10
Y	Trends in Genetics (TIG), volume 4, no. 4, April 1988, Elsevier Publications, (Cambridge, GB), J. Rigden et al.: "Pathogenesis-related proteins in plants", pages 87-89 see page 88, last paragraph	13
Y	Nature, volume 324, no. 6095, 27 November-3 December 1986, (Neptune, NJ, US), A. Schlumbaum et al.: "Plant chitinases are potent inhibitors of fungal growth", pages 365-367 see the whole article	15
A	Journal of Cellular Biochemistry, Supplement 12C, UCLA Symposia on Molecular & Cellular Biology, Abstracts 17th Annual Meetings, 28 February - 10 April 1988, Alan R. Liss, Inc., (New York, US), R. Broglie et al.: "Regulation of chitinase gene expression in transgenic plants", page 149, see page 149, abstract L 045	15,16
A	Proc. Natl. Acad. Sci. USA, volume 83, September 1986, K.E. Broglie et al.: "Ethylene-regulated gene expression- molecular cloning of the genes encoding an endochitinase from Phaseolus vulgaris", pages 6820-6824 see the whole article	15,16
A	Biological Abstracts/RRM, abstract BR36:106155, T.V. Suslow et al.: "Effect of	1-26

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8905501 SA 33569

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/05/90 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0292435	23-11-88	JP-A- 1063373	09-03-89
		ZA-A- 8803552	21-11-88
EP-A- 0289479	02-11-88	AU-A- 1527388	03-11-88
		JP-A- 63287488	24-11-88
EP-A- 0278659	17-08-88	JP-A- 63276492	14-11-88
		ZA-A- 8800319	12-08-88
EP-A- 0157351	09-10-85	US-A- 4751081	14-06-88
EP-A- 0270248	08-06-88	AU-A- 8110487	12-05-88
		JP-A- 63141591	14-06-88

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82